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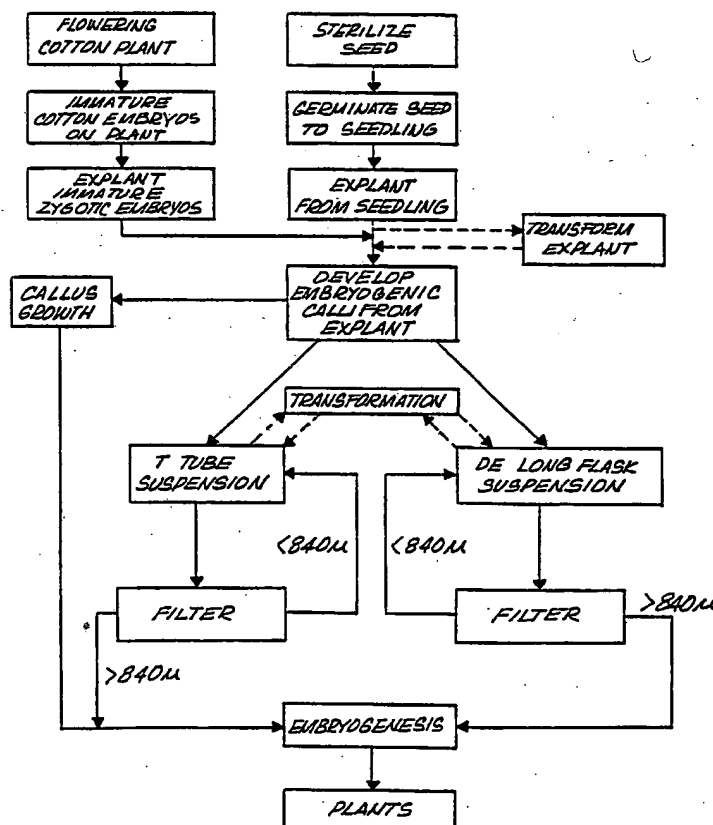
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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## (54) Title: REGENERATION AND TRANSFORMATION OF COTTON

## (57) Abstract

There are provided methods for regenerating cotton by tissue and suspension culture starting with explants which are the hypocotyl, cotyledon or immature embryos. This also taught methods to transform cotton and improve cotton by selective growth.



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REGENERATION AND TRANSFORMATION OF COTTONBACKGROUND OF THE INVENTION

This invention is directed to plant regeneration and transformation of cotton, particularly cotton of the species Gossypium hirsutum L.

In recent years many tissues of diverse origin from plants belonging to different taxonomic groups have been established as in vitro tissue culture. Some of the factors controlling growth and differentiation of such cultures have also been determined. The establishment of subtle interactions among the different groups of plant hormones, and plant growth regulators operating either directly or indirectly, alone or in synergistic combination, have given to some degree an insight into certain interrelationships that may exist among cells, tissues and organs. The information is however by no means complete.

For some time it has been known that plant cell cultures can be maintained in a nondifferentiating proliferative state indefinitely. It has, however, only been recently found that redifferentiation of tissues, organs or whole plant organisms can be experimentally induced. Since the demonstrations by Skoog et al "Chemical regulation of growth and organ formation in plant tissues cultured in vitro," Symp. Soc. Exp. Biol. 11:18-130,

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1 1958, incorporated herein by reference, that the relative  
ratio of a cytokinin to an auxin determines the nature  
of organogenesis in tobacco pith tissue. Reorganization  
or regeneration from callus cultures includes the formation  
5 of shoot primordia or embryos, both of which ultimately  
lead to plantlet development in vitro.

The tendency for organogenesis vs. embryogenesis  
still depends upon the species involved and the presence  
of certain triggering factors which are chemical and/or  
10 physical in nature.

In 1902, Haberlandt "Kulturversuche mit isolierten  
pflanzenzellen," Mat. KI. Kais, Akad, Wiss. Wien 111:62,  
incorporated herein by reference, postulated that plant  
cells possessed the ability to produce entire plants and  
15 predicted that this would someday be demonstrable in  
cell cultures. In 1965, Reinert "Untersuchungen uber  
die morphogenese an Gewebekulturen," Ber. dt. Bot. Ges.  
71:15, and Steward et al, "Growth and organized development  
of cultured cells/II. Organization in cultures grown  
20 from freely suspended cells," Am. J. Bot. 45:705-708,  
working independently, confirmed the occurrence of in  
vitro somatic embryogenesis. Both references are  
incorporated herein by reference. In experimentally  
manipulating somatic embryogenesis it is believed that  
25 two components of the culture media, an auxin and the  
nitrogen source, play crucial roles.

It has also been shown that the process of somatic  
embryogenesis takes place in two stages: first, the  
induction of cells with embryogenic competence in the  
30 presence of a high concentration of auxin; and second,  
the development of embryonic cell masses into embryos in  
the absence of or at a low concentration of auxin.

The induction of organogenesis or embryogenesis  
leads to distinct structural patterns in the callus.  
35 Detailed study of several plant species has enabled

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1 certain generalizations to be made about the developmental pathways leading to shoot, bud or embryo development.

2 The application of tissue culture techniques to the  
3 regeneration of plants via organogenesis or embryogenesis  
4 remains perhaps the most important contribution of basic  
5 studies in morphogenesis to commercial application.

6 Beasley reported the formation of callus in ovule  
7 cultures of cotton in 1971, "In vitro culture of ferti-  
8 lized cotton ovules," Bioscience 21:906:907, 1971, incor-  
9 porated herein by reference. Later, Hsu et al "Callus  
10 induction by (2-chlorethyl) phosphonic (CPA) acid in  
11 cultured cotton ovules," Physiol. Plant 36:150-153,  
12 1976, incorporated herein by reference, observed a  
13 stimulation of growth of calli obtained from ovules due  
14 to the addition of CPA and gibberellic acid to the medium.  
15 Callus cultures from other explants such as (a) leaf  
16 Davis et al., "In vitro culture of callus tissues and  
17 cell suspensions from okra (Hibiscus esculentus) and  
18 cotton (Gossypium hirsutum)," In vitro 9:395-398, 1974,  
19 both incorporated herein by reference; (b) hypocotyl  
20 Schenk et al. "Medium and technique for induction and  
21 growth of monocotyledonous and dicotyledonous plant cell  
22 cultures," Can. J. Bot. 50:199-204, 1972, incorporated  
23 herein by reference; and (c) cotyledons Rani et al.  
24 "Establishment of Tissue Cultures of Cotton," Plant Sci.  
25 Lett. 7:163-169, 1976, incorporated herein by reference)  
26 have been established for Gossypium hirsutum and G.  
27 arboreum.

28 Katterman et al, "The influence of a strong reducing  
29 agent upon initiation of callus from the germinating  
30 seedlings of Gossypium barbadense," Physiol. Plant 40:98-  
31 101, 1977, incorporated herein by reference, observed  
32 that the compact callus from cotyledons of G. barbadense  
33 formed roots, and in one instance regeneration of a  
34 complete plant was also obtained. Smith et al "Defined  
35

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1 conditions for the initiation and growth of cotton callus  
in vitro, Gossypium arboreum," In vitro 13:329-334,  
1977, incorporated herein by reference, determined  
5 conditions for initiation and subculture of hypocotyl-  
derived callus of G. arboreum. Subsequently, Price et  
al "Callus cultures of six species of cotton (Gossypium  
L) on defined media," Pl Sci. Lett. 8:115-119, 1977, and  
10 "Tissue culture of Gossypium species and its potential  
in cotton genetics and crop improvement," Beltwide Cotton  
Production Research Conference Proc. pp. 51-55, 1977, of  
the National Cotton Council, Memphis, each incorporated  
herein by reference, defined conditions for the initiation  
and subculture of callus from five species of Gossypium.

15 One of the common problems in establishing cultures  
of many plant species is the "browning" of the explant  
in the culture medium. In cotton, this leaching of  
polyphenols was overcome by replacing sucrose with glucose,  
and by transferring the cultures to a fresh medium every  
10 days. After 3 or 4 passages on glucose supplemented  
20 medium, the browning completely disappeared and the  
cultures could be transferred back to sucrose-supple-  
mented media. Although difficulties with the induction,  
browning and maintenance of calli during subcultures  
have been overcome with certain Gossypium species, all  
25 attempts to regenerate plants from callus cultures have  
been either unsuccessful or have involved several time-  
consuming steps. Davidonis and Hamilton "Plant  
Regeneration from Callus Tissue of Gossypium hirsutum,"  
L. Plant Sci. Lett. 32:89-93, 1983, incorporated herein  
30 by reference, reported the eventual formation of embryos  
two years after the initiation of culture.

Although many growth substances, such as natural  
phytohormones and synthetic growth regulating compounds  
have been utilized in tissue culture media to bring  
35 about plant regeneration in vitro, no generalization,

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1 much less specifics, of the effects of different substances  
on plant regeneration has been arrived at. Indeed, the  
same substances, when applied to different plant species,  
may either inhibit growth, enhance growth, or have no  
5 effect whatsoever. Therefore, aside from certain standard  
procedures, it remains necessarily a difficult task to  
arrive at a working protocol for plant regeneration for  
any new species and by many orders of magnitude a more  
difficult task to achieve plant transformation.

10 The present invention provides a method for the  
rapid regeneration of cotton plants from segments excised  
from seedlings. The method described offers a high degree  
of repeatability and reliability and it enables genetic  
transformation of cotton plants.

15

#### Summary of the Invention

There is provided methods for the regeneration,  
with optional transformation, of a cotton plant from  
somatic cells.

20 Seed is sterilized and grown in the dark to a  
seedling. The seedling is one source of an explant,  
usually the hypocotyl and the cotyledon. Another source  
are immature zygotic embryos of developing fruit. The  
explant is subdivided and cultured in a first callus growth  
25 medium (containing glucose) for a period of time to  
allow a callus to develop from the explant on a culture  
medium which copes with phenolic secretions and stimulates  
cells of the explant to divide and proliferate. The  
callus, after passing through the phenolic secretion  
30 stage, is transferred to a fresh callus growth medium  
(containing sucrose) which develops the callus to an  
embryogenic callus. The embryo may then be subcultured  
to produce more embryogenic callus or transferred to  
another growth medium (plant germination medium) and  
35 cultured for a period of time sufficient to develop a



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1     plantlet which after another period of growth is  
transferred to a greenhouse, then into the field and  
grown to a mature plant from which seeds can be harvested.

5     The embryos may also be cultured in suspension. In  
this procedure, after the period of growth, the embryo  
containing embryogenic clumps greater than about 600  
microns, preferably greater than about 800 microns in  
size are isolated and utilized for plant production.  
Smaller callus are recycled to the callus growth medium  
10    for growth to plant forming callus or maintained as an  
embryos source.

Transformation may occur at the explant, callus or  
suspension development stage. Transformation involves  
exposing the explant, callus and/or embryogenic callus  
15    to the action of an Agrobacterium vector containing an  
expressible gene sequence foreign to cotton for a time  
sufficient for the gene to transfer into the cells. The  
residual Agrobacterium is then killed off with an  
antibiotic which is toxic to the Agrobacterium. This is  
20    followed by selection of the transformed cells and/or  
embryogenic callus for development into transformed  
plantlets. In suspension culture, transformation and/or  
selection can occur prior to or following separation of  
embryogenic callus from cells and callus too immature to  
25    be embryogenic.

Plants of unique phenotypic traits are obtainable,  
and there is provided new cotton plants which having  
resistance to antibiotics normally inhibitory to plant  
cell growth; cotton plants which have increased resistance  
30    or tolerance to herbicides, fungal pathogens and cotton  
plants which exhibit better yield and improved fiber  
quality.

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1     Brief Description of the Drawings

FIG. 1 presents diagrammatically preferred procedures for development of cotton plants from seed by tissue culture techniques with a showing of establishing zones of transformation.

FIG. 2 is a photo illustration of embryogenic callus (10) of cotton with somatic embryos (12) at various stages of development including leaf (14) and root (16).

FIG. 3 is a photo illustration of a somatic cotton embryo at a late globular stage isolated to form the embryogenic callus culture as depicted in FIG. 2.

FIG. 4, as with reference to FIG. 2, is a photo illustration of embryos and young plantlets (18) of cotton developing on an embryo germination medium.

FIG. 5 is a photo illustration of small clumps of embryogenic cells from suspension cultures of cotton.

FIG. 6 is a photo illustration of a globular stage embryo from a suspension culture.

FIG. 7 illustrates germinating embryos obtained from suspension cultures showing emerging leaves (14) and roots (16).

FIG. 8 illustrates the development of plantlets of cotton growing on the embryo germination medium.

FIGS. 9 to 15 depict the genetic transformation of cotton, with FIG. 9 showing the development of cell colonies (20) from transformed cotton cells containing a gene for kanamycin resistance.

FIG. 10 shows somatic embryos developing from the selected antibiotic resistance cells of FIG. 9 on an antibiotic-supplemented medium.

FIG. 11 shows germinating embryos of transformed somatic embryos containing a gene conferring resistance to the herbicide glyphosate.

FIG. 12 shows cotton plantlets developed from the embryos of FIG. 11.

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1           FIG. 13 shows germinating somatic embryos transformed to confer resistance to Lepidopterous insects with leaf 14 and root 16 development.

5           FIG. 14 shows plantlets developed from the embryos of FIG. 13.

          FIG. 15 shows a plantlet of the variety Siokra developed from transformed embryos exhibiting a resistance to kanamycin.

10          FIG. 16 shows the construction of mp 19/bt, a plasmid containing the 5' end of the Bt protoxin gene.

          FIG. 17 shows the construction of mp 19/bt ca/del, a plasmid containing the CaMV gene VI promotor fused to the 5' end of Bt protoxin coding sequence.

15          FIG. 18 shows the construction of p702/bt, a plasmid having the 3' coding region of the protoxin fused to the CaMV transcription termination signals.

          FIG. 19 shows the construction of pBR322/bt 14, containing the complete protoxin coding sequence flanked by CaMV promotor and terminator sequences.

20          FIG. 20 shows the construction of pRK252/Tn903/BglIII.

          FIG. 21 shows the construction of PCIB 5.

          FIGS. 22 & 23 shows the construction of pCIB 4.

          FIG. 24 shows the construction of pCIB 2.

25          FIG. 25 shows the construction of pCIB 10, a broad host range plasmid containing T-DNA borders and gene for plant selection.

          FIG. 26 shows the construction of pCIB10/19Sbt.

          FIG. 27 shows the construction of pCIB 710.

          FIG. 28 shows the construction of pCIB10/710.

30          FIG. 29 shows the construction of pCIB10/35Sbt.

          FIG. 30 shows the construction of pCIB10//35Sbt(KpnI).

          FIG. 31 shows the construction of pCIB10/35Sbt(BclI).

          FIG. 32 shows the construction of pCIB10/35Sbt(607)

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1           FIG. 33 depicts the vector DEI PEP10.

FIG. 34 is a photo showing a field trial made up of cotton regenerants planted in a Verticillium infested field.

5           FIG. 35 is a photo showing progeny of a regenerated SJ4 plant in the field trial shown in FIG. 33. A somaclonal variant with improved tolerance to Verticillium fungus is indicated by the arrow.

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1 Detailed Description

The present invention is directed to the regeneration by tissue culture of cotton plants particularly plants of the genus Gossypium hirsutum from somatic cells for propagation in the field. Optionally, the cells may be transformed to include foreign genetic information.

The various growth medium useful in accordance with this invention are as follows:

10

## SEED GERMINATION GROWTH MEDIUM

COMPOSITION OF MODIFIED WHITE'S STOCK SOLUTION  
(Phytomorphology 11:109-127, 1961  
incorporated herein by reference)

15	<u>Component</u>	<u>Concentration per 1000 ml.</u>	<u>Comments</u>
	MgSO <sub>4</sub> .7 H <sub>2</sub> O	3.6 g	Dissolve and make up the final volume to 1000 ml. Label <u>White's A Stock</u> . Use 100 ml/l of final medium.
	Na <sub>2</sub> SO <sub>4</sub>	2.0 g	
	NaH <sub>2</sub> PO <sub>4</sub> .H <sub>2</sub> O	1.65 g	
20			
	Ca(NO <sub>3</sub> ) <sub>2</sub> .4 H <sub>2</sub> O	2.6 g	Dissolve and make up the final volume to 1000 ml. Label <u>White's B Stock</u> . Use 100 ml/l of final medium.
	KNO <sub>3</sub>	800 mg	
	KCl	650 mg	
25			
	Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	2.5 mg	Dissolve and make up the final volume to 100 ml. Label <u>White's C Stock</u> . Use 1.0 ml/l of final medium.
	CoCl <sub>2</sub> .6H <sub>2</sub> O	2.5 mg	
	MnSO <sub>4</sub> .H <sub>2</sub> O	300 mg	
	ZnSO <sub>4</sub> .7 H <sub>2</sub> O	50 mg	
30	CuSO <sub>4</sub> .5 H <sub>2</sub> O	2.5 mg	
	H <sub>3</sub> BO <sub>3</sub>	50 mg	
	Fe EDTA		Use 10 ml/l of MSFe EDTA.
	Organic		Use 10 ml/l of MS organic.
35			

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## CALLUS GROWTH/MAINTENANCE MEDIUM

## COMPOSITION OF MURASHIGE &amp; SKOOG (MS)

## STOCK SOLUTIONS

(Physiol. Plant 15:473-497, 1962  
incorporated herein by reference)

5

	<u>Component</u>	<u>Concentration per 1000 ml. of Stock</u>		<u>Comments</u>
10	NH <sub>4</sub> NO <sub>3</sub>	41.26	g	Dissolve and make up the final volume to 1000 ml. Use 40 ml/l of final medium.
	KNO <sub>3</sub>	47.50	g	
	CaCl <sub>2</sub> .2 H <sub>2</sub> O	11.00	g	
	MgSO <sub>4</sub> .7 H <sub>2</sub> O	9.25	g	
	KH <sub>2</sub> PO <sub>4</sub>	4.25	g	
15	KI	83	mg	Dissolve and make up the final volume to 1000 ml. Label <u>MS -</u> <u>Minor</u> . Use 10 ml/l of final medium.
	H <sub>3</sub> BO <sub>3</sub>	620	mg	
	MnSO <sub>4</sub> . H <sub>2</sub> O	1690	mg	
	ZnSO <sub>4</sub> .7 H <sub>2</sub> O	860	mg	
	Na <sub>2</sub> MoO <sub>4</sub> .2 H <sub>2</sub> O	25	mg	
	CuSO <sub>4</sub> .5 H <sub>2</sub> O	2.5	mg	
	CoCl <sub>2</sub> .6 H <sub>2</sub> O	2.5	mg	
20	Nicotinic acid	50	mg	Dissolve and make up the final volume to 1000 ml. Label <u>MS -</u> <u>Organic</u> . Freeze in 10 ml aliquots. Use 10 ml/l of final medium.
	Pyridoxin HCl	50	mg	
	Thiamine HCl	10	mg	
25				
30				
35				

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1				
	Fe EDTA	2.78	g	
	Fe SO <sub>4</sub> .7H <sub>2</sub> O	3.73	g	
	Na <sub>2</sub> EDTA.2 H <sub>2</sub> O			
5				Dissolve 2.78 g of FeSO <sub>4</sub> .7 H <sub>2</sub> O in about 200 ml of deionized water. Dissolve 3.73 g of Na <sub>2</sub> EDTA.2 H <sub>2</sub> O (disodium salt of ethylenediaminetetraacetic acid dihydrate) in 200 ml of deionized water in another beaker. Heat the Na <sub>2</sub> EDTA solution on a hot plate for about 10 minutes. While constantly stirring, add FeSO <sub>4</sub> solution to Na <sub>2</sub> EDTA solution. Cool the solution to room temperature and make up the volume to 1000 ml. Label <u>MS EDTA</u> . Cover bottle with foil and store in refrigerator. Use 10 ml/l of final medium.
10				
15				
20	Thiamine HCl	50	mg	Dissolve and make up the volume to 500 ml. Label <u>MS - Thiamine</u> . Use 4.0 ml/l of final medium. As if required.
25				
	Inositol	10	g	
	Glycine	0.2	g	
30				Dissolve and make up the final volume to 1000 ml. Label <u>MS - glycine/inositol</u> . Use 10 ml/l of final medium.
35				

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## PLANT GERMINATION MEDIUM

COMPOSITION OF BEASLEY AND TING'S STOCK SOLUTIONS .  
 (Am. J. Bot. 60:130-139, 1973  
 incorporated herein by reference)

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	<u>Component</u>	<u>Concen- tration per 1000 ml.</u>	<u>Comments</u>
10	KH <sub>2</sub> PO <sub>4</sub> H <sub>3</sub> BO <sub>3</sub> Na <sub>2</sub> MoO <sub>4</sub> .2 H <sub>2</sub> O	2.72 g 61.83 mg 2.42 mg	Dissolve and make up the volume to 100 ml. Label <u>B&amp;T - A Stock</u> . Use 10 ml/l of final medium.
15	CaCl <sub>2</sub> .2 H <sub>2</sub> O KI CoCl <sub>2</sub> .6 H <sub>2</sub> O	4.41 g 8.3 mg 0.24 mg	Dissolve and make up the volume to 100 ml. Label <u>B&amp;T - B Stock</u> . Use 10 ml/l of final medium.
20	MgSO <sub>4</sub> .7 H <sub>2</sub> O MnSO <sub>4</sub> .H <sub>2</sub> O ZnSO <sub>4</sub> .7 H <sub>2</sub> O CuSO <sub>4</sub> .5 H <sub>2</sub> O	4.93 g 169.02 mg 86.27 mg 0.25 mg	Dissolve and make up the volume to 100 ml. Label <u>B&amp;T - C Stock</u> . Use 10 ml/l of final medium.
	KNO <sub>3</sub>	25.275 g	Dissolve and make up the volume to 200 ml. Label <u>B&amp;T - D Stock</u> . Use 40 ml/l of final medium.
25	Nicotinic acid Pyridoxin HCL Thiamine HCL	4.92 mg 8.22 mg 13.49 mg	Dissolve and make up the final volume to 100 ml. Label <u>B&amp;T - Organics</u> . Use 10 ml/l of final medium.
30	Fe EDTA		Use 10 ml/l of MS Fe EDTA.
	Inositol		100 mg/l of final medium.
	NH <sub>4</sub> NO <sub>3</sub> (15 uM)		1200.6 mg/l of final medium.

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1           With any of the above solutions, the following  
procedure is used to prepare one liter of the medium.  
There is provided as a base, 200 ml of deionized water  
and the various stock solutions are added in the amounts  
5           stated for 1 liter. For example, if there is to be  
employed 10 ml of a stock in the final medium, then 10  
ml of the stock are added to the 200 ml of the distilled  
water. To ensure the salts stay in solution, stock  
10           solutions are normally added in the order shown in the  
formulations above. After thoroughly mixing additional  
deionized water is added to the mixture to bring it to,  
as required 500 ml, and the mixture adjusted in pH to a  
value of from about 5.8 to 6.0. The final volume is  
15           brought to 1,000 ml and there is normally added tissue  
culture Agar, or its equivalent to a level of about 0.8%  
by weight. This is to provide some solidity to solution  
to reduce flow. The mixture is then autoclaved for about  
5 to 20 minutes at a pressure 15-21 lbs/in<sup>2</sup> to kill any  
contaminating organism, and suitably labeled and stored  
20           as a sterile medium.

Briefly, cotton seeds are sterilized and germinated  
on a suitable seed germination medium such as a basal  
agar medium in the dark for a time sufficient to produce  
seedlings. The normal period of growth is up to about 4  
25           weeks, typically 7 to 14 days.

Segments of explants are excised from the seedling.  
It is preferred that the explant come from the hypocotyl  
or cotyledon. In the alternative, one can equally use  
immature embryos obtained from the developing fruits of  
30           greenhouse or field grown cotton plants as the explant.  
The explant segments are cultured on a suitable first  
callus growth medium, preferably a or full Murashige and  
Skoog (MS) nutrient medium containing glucose. Growth  
occurs by culturing at a temperature of from about 25 to  
35           about 35°C in a light/dark cycle of about 16 hours of

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1 light and above 8 hours of dark. Culturing is the  
procedure whereby the medium is replaced at periodic  
intervals as the nutrients are consumed and continued  
for approximately about 3 to about 4 weeks, or until  
5 undifferentiated callus are formed. The callus are  
transferred to a second callus growth medium, preferably  
an MS medium supplemented with naphthaleneacetic acid  
(NAA) and sucrose as the carbon source and cultured for  
three to four months to produce embryos.

10 The embryos may then be maintained in the second  
callus growth medium to maintain an embryo supply or  
transferred to a plant germination medium such as Beasley  
and Ting's medium preferably containing casein hydrolysate  
and source of ammonium cultured for 2 to 3 weeks to  
15 produce plantlets.

The plantlets are transferred to soil under high  
humidity conditions, then transplanted to larger pots in  
a greenhouse and finally transferred to the field for  
growth to maturity.

20 The methods briefly described herein have been  
successfully employed to induce somatic embryo formation  
in cotton of the species Gossypium hirsutum by tissue  
and suspension cultures and, ultimately, to obtain mature  
plants from hypocotyl and cotyledon derived callus cultures  
25 of Acala varieties of Gossypium hirsutum including SJ2,  
SJ4, SJ5, B1644, B1810, B2724, GC510 and C1 and non  
Acala "picker" Siokra and "stripper" variety FC 2017.  
Cultures have been transformed to normal plants with  
novel traits or properties.

30 More particularly, the procedure involves first the  
sterilizing of the cotton seeds. Suitable sterilization  
may be achieved by immersing the seeds in 95% ethanol  
for 2 to 3 minutes, rinsing in sterile water one or more

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1 times, then soaking the seeds in a 15% solution of sodium  
hypochlorite for 15 to 20 minutes, and rinsing several  
times with sterile water.

5 The sterilized seeds are then transferred to a  
first medium, termed a seed germination medium. A seed  
germination medium is one of normal salt content. A  
suitable germination medium is a basal agar medium,  
including White's medium or half-strength MS medium.  
(One-half ingredient strength). Germination normally  
10 occurs in the dark over an about 12 to about 14 day  
period.

Hypocotyl and/or cotyledons are preferably excised  
from the germinated seed, subdivided or cut into segments  
and cultured on a first callus growth medium such as an  
15 MS medium supplemented with growth substances. The  
presently preferred medium is the MS medium supplemented  
with about 0.4 mg/l thiamine hydrochloride, about 30 g/l  
glucose, about 2 mg/l naphthaleneacetic acid, about 1  
mg/l kinetin, a common growth regulator, and about 100  
20 mg/l inositol and agar. Thiamine hydrochloride can  
generally range in concentration from 0.1 to about 0.5  
mg/l, glucose about 20 to about 30 g/l, about 1 to about  
10 mg/l naphthaleneacetic acid, about 1 to about 2 mg/l  
kinetin and about 50 to about 100 mg/l inositol.

25 The cultures are maintained at a temperature of  
about 25 to about 35°C, preferably about 30°C and with a  
light/dark cycle of about 16 hours of light and about 8  
hours of dark. It is preferred to have a light intensity  
of about 2000 to 4000 lux, more preferably about 3000 to  
30 4000 lux.

The calli formed are periodically subcultured at 3  
to 4 week intervals and transferred to a fresh first callus  
growth medium. In the culturing of the explants, secre-  
tions of phenolic compounds from the explants can occur  
35 as evidenced by darkening of the cultured medium. In

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1 this instance, the medium is changed more regularly.  
Darkening has been avoided by changing the culture medium  
every 10 days. Normally, after three to five medium  
changes, phenolic secretions will disappear. When this  
5 occurs, the first callus growth medium can be replaced  
by fresh callus growth medium containing sucrose or sup-  
plemented with sucrose as a carbon source.

After 3 to 4 weeks of culture, active calli develop  
on the cut surfaces of the explants. The calli are then  
10 transferred to a fresh second callus growth maintenance  
medium which is preferably an MS medium combined with  
about 1 to about 10 mg/l, preferably about 1 to about 5  
mg/l NAA. Cytokinin is employed at a concentration of  
from 0 to about 1 g/l. A callus growth medium is  
15 characterized as a high salt content medium containing  
as much as 10 times more salt than the seed germination  
medium. The essential difference between first and  
second callus growth medium is the carbon source. Glucose  
is used during period of phenolic secretions. Sucrose  
20 is used when secretion have stopped. The balance of the  
callus growth medium can remain the same or changed.

The calli are transferred in regular intervals to a  
fresh callus growth medium and, after generally about 5  
to 7 passages or until an anthocyanin pigmentation becomes  
25 evident in a portion of the calli, which is followed by  
development of a yellowish-white embryogenic callus.

The embryogenic callus are then selectively  
subcultured and maintained by regular subculturing. The  
embryogenic callus contain somatic embryos at various  
30 stages of development. Some may have reached the point  
of development that enables growth into small plantlets.  
Most, however, require further development. Some may be  
advanced to germination. Other may be maintained as a  
source of embryos for future use.

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1 With reference to FIG. 2, there is illustrated this  
stage of development showing calli of Acala cotton 10  
with somatic embryos 12 of differing size with some  
having emerging leaves 14 and roots 16. FIG. 3 illustrates  
5 a somatic embryo isolated at a late globular stage.

With reference to FIG. 4, further development may  
be achieved by transferring the somatic embryos to a  
third growth medium termed herein an embryo germination  
medium, a medium rich in nitrogen usually in the form of  
10 ammonia or its equivalent. Suitable media include Beasley  
and Ting's medium, preferably supplemented with up to about  
500 mg/l casein hydrolysate.

Germination occurs from somatic embryos and, within  
2 to 3 weeks, a well developed plantlet 18 of up to 6  
15 leaves and good root system is generally formed.

At this stage, the plantlets are transferred to  
soil in small clumps and grown in a standard incubator  
under conditions of high humidity. Temperature is normally  
maintained at about 25 to 30°C (See Fig. 7).

20 After a period of growth, the small plants are  
transferred to larger pots in a greenhouse and thereafter  
transferred to field and grown to maturity. All the  
regenerated plants are preferably self-pollinated either  
while growing in the green house or in field conditions  
25 and the seeds collected. Seeds are then germinated and  
4 to 5 week old seedlings transferred to the field for  
progeny row trials and other standard plant breeding  
procedures. Practicing the above procedure produces  
viable cotton plants from about 35% of the explants in  
30 the period of time from about 6 to about 8 months.

#### Proliferation of Embryogenic Cotton Cells In Suspension Cultures

As an alternative to allowing the growing embryogenic  
calli to be developed into a plant, the callus may be cut  
35

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1 into smaller pieces and further developed using suspension  
culture techniques.

2 In this procedure, suspension concentration is  
normally from about 750 to 1000 mg of callus parts to 8  
5 ml. callus growth medium such as the second callus growth  
medium (MS medium supplemented with NAA), and allowed to  
grow in suspension. In a preferred embodiment, the  
suspension of the callus is inserted in T-tubes and  
placed on a roller drum rotating at about 1.5 rpm under  
10 a light regime of about 16 hours of light and about 8  
hours of dark. Growth is for about 3 to 4 weeks.

After about every 3 to 4 weeks, the suspension is  
filtered to remove large cell clumps of embryogenic callus  
depicted in groups in FIG. 5 and as isolated at late  
15 globular stages as shown in FIG. 6. The filtrate is  
returned to a nutrient medium for a 3 to 4 week period of  
growth. This procedure is repeated over and over with  
harvesting of large clumps at about 3 to 4 week intervals,  
at which time the medium is supplanted in whole or in  
20 part with fresh callus growth medium. Preferably, about  
4 volumes or more of the fresh medium are added to about  
one volume of residual suspension. It is presently  
preferred that the filter employed have a mesh size  
greater than about 600 microns, preferably greater than  
25 800 microns, as it has been observed the cell masses of  
a particle size less than 600 microns will not develop  
into plants, whereas cell masses greater than 600 microns  
and preferably greater than 800 microns have undergone  
sufficient differentiation so as to become embryogenic  
and capable of developing into viable plants.  
30

Suspension cultures can also be initiated by trans-  
ferring of embryogenic calli to a flask, such as a DeLong  
or Erlenmeyer flask, containing the liquid embryo growth  
medium in an amount of about 20 ml of MS and NAA at a  
35 concentration of 2.0 mg/l. The flask is placed on a

-20-

1 gyrotory shaker and is shaken at about 100-110 strokes  
per minute. After 3 to 4 weeks the suspension is suitable  
for filtration as described above to remove the large  
cell clumps for plant development.

5 More typically, after the third or fourth subcul-  
ture, the cell suspension from the "T" tube or De Long  
or Erlenmeyer flask is plated onto agar-solidified MS  
medium containing NAA (2.0 mg/l) or Beasley & Ting's medium  
10 containing casein hydrolysate (500 mg/l) media and a  
source of nitrogen. Within 3-4 weeks embryogenic calli  
with developing embryos become visible. Likewise, the  
larger cell clumps when plated on the above media give  
rise to embryogenic clumps with developing embryos.

15 In both suspension growth methods, the MS media is  
used to promote and/or sustain embryos whereas the  
germination medium is employed for rapid plant development.

The seedling explants, if desired, can be transformed.  
In this procedure, cotyledon and/or hypocotyl segments of  
the sterilized seed can be used. Cotyledons are preferred.

20 The segments are placed in a medium containing an  
Agrobacterium vector containing a code (genetic marker)  
such as resistance to an antibiotic, such as for instance  
kanamycin for a time sufficient for the vector to transfer  
the gene to the cells of the explant. Generally, contact  
25 times ranging from 1 minute to 24 hours may be used and  
may be accompanied with intermittent or gentle agitation.  
The explants are then removed and placed on agar-solidified  
callus growth medium such as a MS medium supplemented  
with NAA (2 mg/l) and incubated about 15 to 200 hours at  
30 25 to 35°C, preferably 30°C, on a 16:8 hour light: dark  
regime.

After incubation, the explants are transferred to  
the same medium supplemented with the antibiotic cefotaxime  
preferably in a concentration of 200 mg/l. Cefotaxime  
35 is included to prevent any remaining Agrobacterium from

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1 proliferating and overgrowing the plant tissues.  
Alternatively, the explants can be rinsed with MS medium  
supplemented with NAA (2mg/l) and incubated an additional  
4 to 28 days before rinsing, then incubating the same  
5 medium containing cefotaxime. At the end of 4-5 weeks  
of culture on fresh medium, the developing callus, i.e.,  
primary callus, is separated from the remainder of the  
primary explant tissue and transferred to MS medium  
containing NAA (2 mg/l), cefotaxime (200 mg/l) and an  
10 antibiotic such as kanamycin sulfate (50 mg/l).  
Transformed primary callus, identified by virtue of its  
ability to grow in the presence of the antibiotic  
(kanamycin), is selected and embryos developed, germinated  
and plants obtained following the procedure set forth  
15 above.

It is also feasible to achieve transformation of a  
cell suspension. Following a normal subculture growth  
cycle of 7 to 14 days, usually 7 to 10 days, cells are  
allowed to settle leaving a supernatant which is removed.  
20 The remaining concentrated suspended cells may be  
centrifuged at 4000Xg for 5 minutes and the excess medium  
is discarded. The concentrated suspension cultures are  
resuspended in the 8 ml of the same medium which contains  
the Agrobacterium. The suspension is transferred to "T"  
25 tubes and suitably agitated for incubation.

Following about 2 to 24 hours, preferably 3 to 5  
hours, of incubation to allow for bacterial attachment and  
DNA transfer, the suspension is removed and allowed to  
settle. The supernatant containing the bacteria is  
30 discarded and the cells are washed with fresh medium.  
The suspension may, if desired, be centrifuged for about  
5 minutes and the supernatant removed. In either event,  
the cells are resuspended in the same medium and  
transferred to a "T" tube or flask and suspension subcul-  
35 ture resumed. The object is to minimize the amount of  
unattached Agrobacterium vector left in the cell suspension.



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1           After about 15 to about 200 hours, typically 15 to  
about 72 hours, preferably 18 to 20 hours, the suspension  
is filtered to remove large clumps and washed with fresh  
liquid medium and allowed to settle. The suspension is  
5           resuspended in the fresh liquid medium containing  
cefotaxime (200 mg/l) plated on a solidified medium in  
Petri dishes.

          Alternatively, the suspension may be resuspended in  
fresh medium containing cefotaxime and allowed to grow  
10          an additional 4 to 28 days prior plating on solidified  
medium in Petri dishes. Cell concentration is 1 vol. of  
suspension cells plus 3 vol. of medium with cefotaxime.  
Kanamycin at 10 to 300 mg/l preferably about 20 to 200  
mg/l more preferably about 40 to 80 mg/l is included in  
15          the medium for selection of transformed cells expressing  
the neomycin phosphotransferase (NPT) gene. Cells and  
embryos proliferating in the selective concentration of  
kanamycin are further grown as set forth above to mature  
somatic embryos capable of germinating and regenerating  
20          into whole plants according to the procedures described  
herein.

          Using the above procedure and with reference to  
FIG. 9, there is shown variable cell colonies which is  
consequence of transformation. There exists cotton  
25          cells 20 exhibiting resistance to the antibiotic kanamycin.  
With reference to FIG. 10, transformed calli are shown  
developing into somatic embryos on an antibiotic MS  
medium. FIG. 11 shows transformed somatic embryos  
established to have kanamycin resistance and transformed  
30          to have resistance to the herbicide glyphosate. FIG. 12  
shows plants from the embryos of FIG. 11. FIG. 13 shows  
cells transformed to have resistance to lepidopterous  
insects growing on an MS medium and in FIG. 14 trans-  
ferred to a Beasley and Ting's medium whereas FIG. 15  
35          shows further development of the plantlets of FIG. 14 to  
more mature plantlets.

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1

COTTON REGENERATIONExample 1

5

Regeneration of plants starting  
from cotyledon explants

Seeds of Acala cotton variety SJ2 of Gossypium  
hirsutum were sterilized by contact with 95% alcohol for  
three minutes, then twice rinsed with sterile water and  
10 immersed with a 15% solution of sodium hypochlorite for  
15 minutes, then rinsed in sterile water. Sterilized  
seeds were germinated on a basal agar medium in the dark  
for approximately 14 days to produce a seedling. The  
cotyledons of the seedlings were cut into segments of  
15 2-4mm<sup>2</sup> which were transferred aseptically to a callus  
inducing medium consisting of Murashige and Skoog (MS)  
major and minor salts supplemented with 0.4 mg/l thiamine-  
HCl, 30 g/l glucose, 2.0 mg/l naphthaleneacetic acid  
(NAA), 1 mg/l kinetin, 100 mg/l of m-inositol, and agar  
20 (0.8%). The cultures were incubated at about 30°C under  
conditions of 16 hours light and 8 hours darkness in a  
Percival incubator with fluorescent lights (cool daylight)  
providing a light intensity of about 2000-4000 lux.

Calli were formed on the cultured tissue segments  
25 within 3 to 4 weeks and were white to gray-greenish in  
color. The calli formed were subcultured every three to  
four weeks onto a callus growth medium comprising MS  
medium containing 100 mg/l m-inositol, 20 g/l sucrose, 2  
mg/l naphthaleneacetic acid (NAA) and agar. Somatic  
30 embryos formed four to six months after first placing  
tissue explants on a callus inducing medium. The callus  
and embryos were maintained on a callus growth medium by  
subculturing onto fresh callus growth medium every three  
to four weeks.

35

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1           Somatic embryos which formed on tissue pieces were  
explanted either to fresh callus growth medium, or to  
Beasley & Ting's medium (embryo germination medium).

5           The somatic plantlets which were formed from somatic  
embryos were transferred onto Beasley and Ting's medium  
which contained 1200 mg/l ammonium nitrate and 500 mg/l  
casein hydrolysate as an organic nitrogen source. The  
medium was solidified by a solidifying agent (Gelrite)  
and plantlets were placed in Magenta boxes.

10          The somatic embryos developed into plantlets within  
about three months. The plantlets were rooted with six  
to eight leaves and about three to four inches tall and  
were transferred to soil and maintained in an incubator  
under high humidity for three to four weeks and then  
15       transferred to a greenhouse. After hardening, plants  
were also transferred to open tilled soil.

#### Example 2

20          The procedure of Example 1 was repeated using instead  
half-strength MS medium in which all medium components  
have been reduced to one-half the specified concentration.  
Essentially the same results were obtained.

#### 25                           Example 3

The procedures of Examples 1 and 2 were repeated  
except that the explant was the hypocotyl segments. The  
same results were obtained.

#### 30                           Example 4

The procedure of Examples 1 and 2 were repeated except  
that the explant was the immature zygotic embryo.  
Essentially the same results were obtained.

35

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1 Example 5

The procedure of Examples 1 and 2 was repeated with Acala cotton varieties SJ4, SJ5, SJ2C-1, GC510, B1644, B 2724, B1810, the picker variety Siokra and the stripper variety FC2017. All were successfully regenerated.

### Example 6

10           The procedure of Example 1 was repeated to the  
extent of obtaining callus capable of forming somatic  
embryos. Pieces of about 750-1000 mg of actively growing  
embryogenic callus was suspended in 8 ml units of liquid  
suspension culture medium comprised of MS major and  
15   minor salts, supplemented with 0.4 mg/l thiamine HCl, 20  
g/l sucrose, 100 mg/l of inositol and naphthaleneacetic  
acid (2 mg/l) in T-tubes and placed on a roller drum  
rotating at 1.5 rpm under 16:8 light:dark regime. Light  
intensity of about 2000-4500 lux was again provided by  
20   fluorescent lights (cool daylight).

After four weeks, the suspension was filtered through an 840 micron size nylon mesh to remove larger cell clumps. The fraction smaller than 840 microns were allowed to settle, washed once with about 20-25 ml of fresh suspension culture medium. This suspension was transferred to T-tubes (2 ml per tube) and each tube diluted with 6 ml of fresh suspension culture medium. The cultures were maintained by repeating the above procedure at 10-12 day intervals. Namely, the suspension was filtered and only the fraction containing cell aggregates smaller than 840 microns was transferred to fresh suspension culture medium. In all instances, the fraction containing cell clumps larger than 840 microns was placed onto the callus growth medium to obtain mature somatic embryos.

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1           The somatic embryos that were formed on callus growth  
medium were removed and transferred to embryo germination  
medium and using the protocol of Example 1 were germinated,  
developed into plantlets and then field grown plants.

#### 5                           Example 7

10           The procedure of Example 6 was repeated except that  
suspension cultures were formed by transferring 750-1000  
mg of embryogenic calli to a DeLong flask containing 15-  
20 ml of the MS liquid medium containing 2 mg/l NAA.  
The culture containing flask was placed on a gyrotory  
shaker and shaken at 100-110 strokes/minute. After  
three weeks the suspension was filtered through an 840  
15          micron nylon mesh to remove the large cell clumps for  
plant growth, as in Example 4. The less than 840 micron  
suspension was allowed to settle, washed once in the MS  
liquid medium and resuspended in 2 to 5 ml of the MS  
liquid medium. The suspension was subcultured by transfer  
20          to fresh medium in a DeLong flask containing 1-2 ml of  
suspension and 15 ml of fresh MS liquid medium. The  
cultures are maintained by repeating this procedure at  
seven to ten day intervals. At each subculture only the  
less than 840 micron' suspension was subcultured and the  
25          large clumps (840 microns or greater) were used for plant  
growth.

#### Example 8

30           After three or four subcultures using the suspension  
growth procedure of Examples 6 and 7, 1.5 to 2.0 ml of  
cell suspension from the T-tube and DeLong flask were in  
each instance plated onto agar-solidified MS medium  
containing 2 mg/l NAA and Beasley & Ting medium containing  
35          500 mg/l casein hydrolysate. Within three to four weeks

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1 embryogenic calli with developing embryos became visible.  
Again, the 840 micron or greater cell clumps were plated  
on the callus growth medium giving rise to embryogenic  
clumps with developing embryos which ultimately grew  
5 into plants.

### COTTON TRANSFORMATION

#### Example 9

#### Transformation To Form Tumorous-Phenotype With Agrobacteria LBA 4434

An Acala cotton suspension culture was subcultured  
for three to four months in T-tubes with the medium (MS  
15 medium containing 2 mg/l NAA) being changed every seven  
to ten days. After any medium change thereafter the  
cells can be allowed to settle and harvested for  
transformation. The supernatant was removed by pipeting  
and cells transformed with the Agrobacterium strain LBA  
20 4434. The Agrobacterium strain LBA 4434 is described in  
(Hoekema, A. et al. Nature 303: 179-180, 1983, incorporated  
herein by reference) contains a Ti plasmid-derived binary  
plant transformation system. In such binary systems,  
one plasmid contains the T-DNA of a Ti-plasmid, the  
25 second plasmid contains the vir-region of a Ti-plasmid.  
The two plasmids cooperate to effect plant transformation.  
In the strain LBA 4434, the T-DNA plasmid, pAL 1050,  
contains T<sub>L</sub> of pTiAch5, an octopine Ti-plasmid and the  
vir-plasmid in strain LBA 4434, pAL4404, contains the  
30 intact virulence regions of pTiAch 5 (Ooms, G. et al.  
Plasmid 7:15-29, 1982, incorporated herein by reference).  
Strain LBA 4434 is available from Dr. Robert Schilperoort  
of the Department of Biochemistry, University of Leiden,  
The Netherlands.

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1       The transforming *Agrobacterium* strain was taken  
from a glycerol stock, inoculated in a small overnight  
culture, from which a 50-ml culture was inoculated the  
following day. *Agrobacteria* was grown on YEB medium  
5       containing per liter in water adjusted to pH 7.2 with  
NaOH, 5 g beef extract, 1 g yeast extract, 5 g peptone,  
5 g sucrose. After autoclaving, 1 ml of 2 M  $MgCl_2$  is  
added after which antibiotics, as required to kill other  
strains. The absorbance at 600 nm of the 50 ml overnight  
10       culture is read, the culture centrifuged and the formed  
pellet resuspended in the plant cell growth medium (MS  
medium plus NAA at 2 mg/l) to a final absorbance at 600  
nm of 0.5.

15       Eight ml of this bacterial suspension of *Agrobacterium*  
LBA 4434 was added to each T-tube containing the suspension  
plant cells after removal of the supernatant liquid.  
The T-tube containing the plant and bacteria cells was  
agitated to resuspend the cells and returned to a roller  
drum for three hours to allow the *Agrobacteria* to attach  
20       to the plant cells. The cells were then allowed to  
settle and the residual supernatant removed. A fresh  
aliquot of growth medium was added to the T-tube and the  
suspension allowed to incubate on a roller drum for a  
period of 18 to 20 hours in the presence of any residual  
25       *Agrobacteria* which remained. After this time, the cells  
were again allowed to settle, the supernatant removed and  
the cells washed twice with a solution of growth medium  
containing cefotaxime (200 ug/ml). After washing, the  
cells from each T-tube were resuspended in 10 ml growth  
30       medium containing cefotaxime (200 ug/ml in all cases)  
and 1 ml aliquots of the suspension plated on petri dishes.

35       Infected cells grew on the growth medium to which no  
phytohormones were added establishing the tissue had  
received the wild-type phytohormone genes in T-DNA. The

-29-

1 cells developed tumors, further indicating transformation  
of the cultures.

Example 10

Transformation of Cotton To Form a Kanamycin-Resistant  
5 Non-Tumorous Phenotype

The suspension culture as obtained in Example 9 was transformed using an Agrobacteria which contained the T-DNA containing binary vector pCIB 10. (Rothstein, S.J.  
10 et al. Gene 53: 153-161, 1987, incorporated herein by reference) as well as the pAL 4404 vir-plasmid. The T-DNA of pCIB 10 contains a chimeric gene composed of the promoter from nopaline synthase, the coding region from Tn5 encoding the enzyme neomycin phosphotransferase, and  
15 the terminator from nopaline synthase. The Agrobacteria containing pCIB 10 were grown on YEB medium containing kanamycin (50 ug/ml). Transformation was accomplished in the same manner as in Example 10 except that the 1 ml aliquots resulting in cells and Agrobacteria were  
20 immediately plated on selective media containing either kanamycin (50 ug/ml) or G418 (25 ug/ml). Expression of the nos/neo/nos chimeric gene in transformed plant tissue allows the selection of this tissue in the presence of both antibiotics. The existence in two to four weeks of  
25 transformed tissue became apparent on the selection plates. Uninfected tissue as well as added control tissue showed no signs of growth, turned brown and died. Transformed tissue grew very well in the presence of both kanamycin and G418.

30 At this time, tissue pieces which were growing well were subcultured to fresh selection medium. Somatic embryos formed on these tissue pieces and were explanted to fresh non-selective growth media. When the embryos began to differentiate and germinate, i.e., at the point  
35 where they were beginning to form roots and had two or



-30-

1 three leaves, they were transferred to Magenta boxes  
containing growth medium described in Example 1. Growth  
was allowed to proceed until a plantlet had six to eight  
leaves, at which time it was removed from the agar medium.

5 The plantlets were now placed in potting soil,  
covered with a beaker to maintain humidity and placed in  
a Percival incubator for four to eight weeks. At this  
time, the plant was removed from the beaker and transferred  
to a greenhouse. The plants grew in the greenhouse,  
10 flowered and set seed.

#### Example 11

The procedure of Example 10 was followed, except  
that the transforming Agrobacteria used contained the T-  
15 DNA vector DEI PEP10 as well as the pAL4404 vir plasmid.  
DEI PEP10, shown in Figure 33, utilizes two T-DNA PstI  
cleaved right border sequences from A. Tumefaciens (strain  
C-58) which had been further subdivided with BamHI for  
integration in the plant genome, a passenger maize  
20 phosphoenolpyruvate carboxylase gene (Pepcase gene), and  
a chimeric gene (NOS/NPT/TK) capable of expression in  
plants and conferring resistance to the antibiotics  
kanamycin and G418. This chimeric gene utilizes a nopaline  
synthetase promoter, the neomycin phosphotransferase II  
25 coding region from Tn5, and the terminator from the  
herpes simplex virus thimidine kinase gene. Following  
transformation, embryogenic callus and embryos were  
obtained by selection on kanamycin (50 mg/l). No resistant  
callus was obtained from the control (non-transformed  
30 callus) plated on kanamycin at this level (50 mg/l).

#### Example 12

##### Transformation of Cotton Suspension Culture Cells

##### To A Glyphosate-Tolerant Phenotype

The procedure of Example 10 was followed, except  
35 that the transforming Agrobacteria used contained the T-

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1 DNA vector pPMG85/587 (Fillatti, J. et al., Mol Gen.  
Genet. 206: 192-199, 1987, incorporated herein by  
reference) as well as the pAL4404 vir plasmid. The  
5 plasmid pPMG85/587 carries three chimeric genes capable  
of expression in plants. Two genes code for neomycin  
phosphotransferase (NPT) which confers resistance to the  
antibiotics kanamycin and G418. The third chimeric  
10 gene, containing the coding sequence from a mutant aroA  
gene of *S. typhimurium*, confers tolerance to the herbicide  
glyphosate (Comai, et al., Science 221: 370-371, 1983,  
incorporated herein by reference). The Agrobacteria  
containing pPMG85/587 were grown on medium containing  
kanamycin (100 ug/ml). Transformation is accomplished  
15 as detailed in Example 10 except that the suspension is  
allowed to grow for 28 days at which time 1 ml aliquots  
were plated on medium containing selective antibiotics.  
Expression of the NPT chimeric gene in transformed plant  
tissue allowed selection of this tissue on both  
20 antibiotics. In this instance the selective antibiotic  
was kanamycin (50 ug/ml).

In two to four weeks, transformed tissue became  
apparent on the selection plates. Plant tissue, individual  
embryos and callus were then placed on growth medium  
containing the herbicide glyphosate 1mM and transformed  
25 tissue continued to grow well. Extraction and analysis  
of the proteins of both callus and embryos confirmed the  
presence of the product of the glyphosate tolerance gene.

### Example 13

#### 30 Transformation of Cotton Suspension Culture Cells To a Hygromycin-Resistant Non-Tumorous Phenotype

The transformation procedure of Example 10 was  
followed except there was used as the transforming  
35 Agrobacteria one containing the T-DNA binary vector pCIB

-32-

1 715 (Rothstein, S. J. et al. Gene 53: 153-161, 1987) as  
well as the vir plasmid. The T-DNA of pCIB 715 contains  
a chimeric gene composed of the promoter and terminator  
from the cauliflower mosaic virus (CaMV) 35S transcript  
5 (Odell et al, Nature 313: 810-812, 1985, incorporated  
herein by reference) and the coding sequence for hygromycin  
B phosphotransferase (Gritz, L. and J. Davies, Gene 25:  
179-188, incorporated herein by reference). Agrobacteria  
containing pCIB 715 was grown on YEB containing kanamycin  
10 (50 ug/ml).

Transformation was accomplished as detailed in  
Example 10 again with the change that the 1 ml aliquots  
were plated immediately on medium containing as the  
selective antibiotic 50 ug/ml hygromycin. Expression of  
15 the chimeric hygromycin gene in transformed plant tissue  
allows the selection of this tissue on the medium  
containing hygromycin. Transformed tissue was grown in  
the manner described in Example 8 on the selection growth  
medium establishing transformation had occurred.

20

#### Example 14

##### Transformation of Cotton Suspension Culture Cells To Confer Resistance To Lepidopteran Insects

25 The procedure of Example 10 was followed except where  
changes are noted below. Different transforming  
Agrobacteria were used. Also, after plant tissue was  
selected on an antibiotic for the selection of transformed  
material, it was further selected for expression of the  
BT gene as defined herein.

30 The Agrobacteria used contained the T-DNA vector  
pCIB10 (Rothstein et al, Gene 53:153-161 (198) incorporated  
herein by reference into which had been inserted the  
following chimeric Bacillus thuringiensis endotoxin  
genes ("BT Genes"):

35

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1           To prepare the Agrobacterium vector there was fused  
the CaMV gene VI promotor and protoxin coding sequences.  
A derivative of phage vector mp19 (Yanish-Perron et al.,  
1985) was first constructed. The steps are shown in  
5       FIGS. 16 and 17. First, a DNA fragment containing  
approximately 155 nucleotides 5' to the protoxin coding  
region and the adjacent approximately 1346 nucleotides  
of coding sequence are inserted into mp19. Phage mp19  
ds rf (double-stranded replicative form) DNA was digested  
10       with restriction endonucleases SacI and SmaI and the  
approximately 7.2-kbp vector fragment was purified after  
electrophoresis through low-gelling temperature agarose  
by standard procedures. Plasmid pKU25/4, containing  
approximately 10 kbp (kilobase pairs) of *Bacillus*  
15       *thuringiensis* DNA, including the protoxin gene, was  
obtained from Dr. J. Nueesch, CIBA-Geigy Ltd., Basle,  
Switzerland. The nucleotide sequence of the protoxin  
gene present in plasmid pKU25/4 is shown in Formula 1  
below. Plasmid pKU25/4 DNA was digested with endonucleases  
20       HpaI and SacI, and a 1503 bp fragment containing  
nucleotides 2 to 1505 of Formula 1 and purified. This  
fragment contains approximately 155 bp of bacteria promotor  
sequences and approximately 1346 bp of the start of the  
protoxin coding sequence. Approximately 100 ng of each  
25       fragment is then mixed, T4 DNA ligase added, and incubated  
at 15°C overnight. The resulting mixture was transformed  
into *E. coli* strain HB 101, mixed with indicator bacteria  
*E. coli* JM 101 and plated. One phage (mp19/bt) was used  
for further construction below.

30           Next, a fragment of DNA containing the CaMV gene VI  
promotor, and some of the coding sequences for gene VI,  
was inserted into mp19/bt. Phage mp19/bt ds rf DNA is  
digested with BamHI, treated with the large fragment of  
DNA polymerase to create flush ends and recleaved with  
35       endonuclease PstI. The larger vector fragment was purified

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1 by electrophoresis as described above. Plasmid pABD1 is  
described in Paszkowski et al., EMBO J. 3, 2717-2722,  
(1984) incorporated herein by reference. Plasmid pABD1  
DNA is digested with PstI and HindIII. The fragment  
5 approximately 465 bp long containing the CaMV gene VI  
promotor and approximately 75 bp of gene VI coding sequence  
was purified. The two fragments were ligated and plated  
as described above. One of the resulting recombinant  
phages, mp19/btca contained the CaMV gene VI promotor  
10 sequences, a portion of the gene VI coding sequence,  
approximately 155 bp of *Bacillus thuringiensis* DNA upstream  
of the protoxin coding sequence, and approximately 1346  
bp of the protoxin coding sequence. To fuse the CaMV  
promotor sequences precisely to the protoxin coding  
15 sequenes, the intervening DNA was deleted using  
oligionucleotide-directed mutagenesis of mp19/btca DNA.  
A DNA oligonucleotide with the sequence (5')  
TTCGGATTGTTATCCATGGTTGGAGGTCTGA (3) was synthesized by  
routine procedures using an Applied Biosystems DNA  
20 Synthesizer. This oligonucleotide is complimentary to  
those sequences in phage mp19/btca DNA at the 3' end of  
the CaMV promotor (nucleotides 5762 to 5778 in Hohn,  
Current Topics, in Microbiology and Immunology, 96, 193-  
235 (1982) incorporate herein by reference and the  
25 beginning of the protoxin coding sequence (nucleotides  
156 to 172 in formula I above). The general procedure  
for the mutagenesis is that described in Zoller and Smith,  
Meth, Enzym., 100 468-500 (1983) incorporated herein by  
reference. Approximately five micrograms of single-standed  
30 phage mp19/btca DNA was mixed with 0.3 mg of phosphorylated  
oligonucleotide in a volume of 40 ul. The mixture was  
heated to 65°C for 5 min, cooled to 50°C, and slowly  
cooled to 4°C. Next, buffer, nucleotide triphosphates,  
ATP, T<sub>4</sub> DNA ligase and large fragment of DNA polymerase  
35 were added and incubated overnight at 15°C as described

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1 [Zoller and Smith Meth. Enzym., 100, 468-500 (1983)]  
incorporated herein by reference. After agarose gel  
electrophoresis, circular double-stranded DNA was purified  
and transfected into E. coli strain JM101. The resulting  
5 plaques are screened for sequences that hybridize with  
32P-labeled oligonucleotide, and phage are analyzed by  
DNA restriction endonuclease analysis. Among the resulting  
phage clones were ones which have correctly deleted the  
unwanted sequences between the CaMV gene VI promoter and  
10 the protoxin coding sequence. This phage is called  
mp19/btca/del (see FIG. 17).

Next, a plasmid was constructed in which the 3' coding  
region of the protoxin gene was fused to CaMV transcription  
termination signals. The steps are shown in FIG. 18.  
15 First, plasmid pABDI DNA was digested with endonucleases  
BamHI and BglII and a 0.5 kbp fragment containing the  
CaMV transcription terminator sequences isolated. Next  
plasmid pUC19, Yanisch-Perron et al., Gene, 33, 103-119  
(1985) incorporated herein by reference was digested  
20 with BamHI, mixed with the 0.5 kbp fragment and incubated  
with T<sub>4</sub> DNA ligase. After transformation of the DNA  
into E. coli strain HB101, one of the resulting clones,  
called plasmid p702, was obtained which has the structure  
shown in FIG. 18. Next, plasmid p702 DNA was cleaved  
25 with endonucleases SacI and SmaI, and the larger,  
approximately 3.2 kbp fragment isolated by gel  
electrophoresis. Plasmid pKU25/4 DNA was digested with  
endonucleases AhaIII and SacI, and the 2.3-kbp fragment  
(nucleotides 1502 to 3773 of Formula 1) containing the  
30 3' portion of the protoxin coding sequence (nt 1504 to  
3773) was isolated after gel electrophoresis. These two  
DNA fragments are mixed, incubated with T<sub>4</sub> DNA ligase and  
transformed into E. coli strain HB101. The resulting  
plasmid was p702/bt (FIG. 18).

35

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1           Finally, portions of phage mp19/btca/del ds rf DNA  
and plasmid p702/bt were joined to create a plasmid  
containing the complete protoxin coding sequence flanked  
by CaMV promoter and terminator sequences (see FIG. 18).  
5   Phage mp19/btca/del DNA was digested with endonucleases  
SacI and SphI, and a fragment of approx. 1.75 kbp is  
purified following agarose gel electrophoresis. Similarly,  
plasmid p702/bt DNA is digested with endonucleases SacI  
and SalI and a fragment of approximately 2.5 kbp is  
10 isolated. Finally, plasmid pBR 322 DNA (Bolivar et al.,  
Gene, 2, 95-113 (1977) incorporated herein by reference  
was digested with SalI and SphI and the larger 4.2-kbp  
fragment isolated. All three DNA fragments were mixed  
and incubated with T4 DNA ligase and transformed into E.  
15 coli strain HB101. The resulting plasmid, PBR322/bt14  
is a derivative of PBR322 containing the CaMV gene VI  
promoter and translation start signals fused to the  
Bacillus thuringiensis crystal protein coding sequence,  
followed by CaMV transcription termination signals (shown  
20 in FIG. 19).

The vector pCIB10 is a Ti-plasmid-derived vector  
useful for transfer of the chimeric gene to plants via  
Agrobacterium tumefaciens. The vector is derived from  
the broad host range plasmid pRK 252, which may be obtained  
25 from Dr. W. Barnes, Washington University, St. Louis,  
Mo. The vector also contains a gene for kanamycin  
resistance in Agrobacterium, from Tn903, and left and  
right T-DNA border sequences from the Ti plasmid pTiT37.  
Between the border sequences are the polylinker region  
30 from the plasmid pUC18 and a chimeric gene that confers  
kanamycin resistance in plants.

First, plasmid pRK252 was modified to replace the gene  
conferring tetracycline-resistance with one conferring  
resistance to kanamycin from the transposon Tn903 [Oka,  
35 et al., J. Mol. Biol., 147, 217-226 (1981) incorporated

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1        herein by reference], and was also modified by replacing  
the unique EcoRI site in pRK252 with a BglII site (see  
FIG. 20 for a summary of these modifications). Plasmid  
pRK252 was first digested with endonucleases SalI and  
5        SmaI, then treated with the large fragment of DNA  
polymerase I to create flush ends, and the large vector  
fragment purified by agarose gel electrophoresis. Next,  
plasmid p368 was digested with endonuclease BamHI, treated  
with the large fragment of DNA polymerase, and an  
10       approximately 1050-bp fragment isolated after agarose  
gel electrophoresis; this fragment containing the gene  
from transposon Tn903 which confers resistance to the  
antibiotic kanamycin [Oka et al., J. Mol. Biol., 147,  
217-226 (1981) incorporated herein by reference]. Both  
15       fragments were then treated with the large fragment of DNA  
polymerase to create flush ends. Both fragments are  
mixed and incubated with T4 DNA ligase overnight at  
15°C. After transformation into E. coli strain HB101  
and selection for kanamycin resistant colonies, plasmid  
20       pRK252/Tn903 is obtained (see FIG. 19).

Plasmid pRK252/Tn903 was digested at its EcoRI site,  
followed by treatment with the large fragment of E. coli  
DNA polymerase to create flush ends. This fragment was  
added to synthetic BglII restriction site linkers, and  
25       incubated overnight with T<sub>4</sub> DNA ligase. The resulting  
DNA was digested with an excess of BglII restriction  
endonuclease and the larger vector fragment purified by  
agarose gel electrophoresis. The resulting fragment was  
again incubated with T4 DNA ligase to recircularize the  
30       fragment via its newly-added BglII cohesive ends.  
Following transformation into E. coli strain HB101,  
plasmid pRK252/Tn903/BglII is obtained (see FIG. 20).

A derivative of plasmid pBR322 was constructed which  
contains the Ti plasmid T-DNA borders, the polylinker  
35       region of plasmid pUC19, and the selectable gene for



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1 kanamycin resistance in plants (see FIG. 21). Plasmid  
pBR325/Eco29 contains the 1.5-kbp EcoRI fragment from  
the nopaline Ti plasmid pTiT37. This fragment contains  
5 the T-DNA left border sequence; Yadav et al., Proc.  
Natl. Acad. Sci. USA, 79, 6322-6326 (1982) incorporated  
herein by reference. To replace the EcoRI ends of this  
fragment with HindIII ends, plasmid pBR325/Eco29 DNA was  
10 digested with EcoRI, then incubated with nuclease S1,  
followed by incubation with the large fragment of DNA  
polymerase to create flush ends, then mixed with synthetic  
HindIII linkers and incubated with T4 DNA ligase. The  
resulting DNA was digested with endonucleases ClaI and  
an excess of HindIII, and the resulting 1.1-kbp fragment  
15 containing the T-DNA left border purified by gel  
electrophoresis. Next, the polylinker region of plasmid  
pUC19 was isolated by digestion of the plasmid DNA with  
endonucleases EcoRI and HindIII and the smaller fragment  
(approx. 53 bp) isolated by agarose gel electrophoresis.  
Next, plasmid pBR322 was digested with endonucleases  
20 EcoRI and ClaI, mixed with the other two isolated  
fragments, incubated with T4 DNA ligase and transformed  
into E. coli strain HB101. The resulting plasmid, pCIB5,  
contains the polylinker and T-DNA left border in a  
derivative of plasmid pBR322 (see FIG. 21).

25 A plasmid containing the gene for expression of  
kanamycin resistance in plants was constructed (see  
FIGS. 22 and 23). Plasmid Bin6 obtained from Dr. M.  
Bevan, Plant Breeding Institute, Cambridge, UK. This  
plasmid is described in the reference by Bevan, Nucl.  
30 Acids Res., 12, 8711-8721 (1984) incorporate herein by  
reference. Plasmid Bin6 DNA was digested with EcoRI and  
HindIII and the fragment approximately 1.5 kbp in size  
containing the chimeric neomycin phosphotransferase (NPT)  
gene is isolated and purified following agarose gel  
35 electrophoresis. This fragment was then mixed with plasmid

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1 pUC18 DNA which had been cleaved with endonucleases  
EcoRI and HindIII. Following incubation with T4 DNA  
ligase, the resulting DNA was transformed into E. coli  
strain HB101. The resulting plasmid is called pUC18/neo.  
5 This plasmid DNA containing an unwanted BamHI recognition  
sequence between the neomycin phosphotransferase gene  
and the terminator sequence for nopaline synthase; see  
Bevan, Nucl. Acids Res., 12, 8711-8721 (1984) incorporated  
herein by reference. To remove this recognition sequence,  
10 plasmid pUC18/neo was digested with endonuclease BamHI,  
followed by treatment with the large fragment of DNA  
polymerase to create flush ends. The fragment was then  
incubated with T4 DNA ligase to recircularize the fragment,  
and transformed into E. coli strain HB101. The resulting  
15 plasmid, pUC18/neo(Bam) has lost the BamHI recognition  
sequence.

The T-DNA right border sequence was then added next  
to the chimeric NPT gene (see FIG. 24). Plasmid  
pBR325/Hind23 contains the 3.4-kbp HindIII fragment of  
20 plasmid pTiT37. This fragment contains the right T-DNA  
border sequence; Bevan et al., Nucl. Acids Res., 11,  
369-385 incorporated herein by reference. Plasmid  
pBR325/Hind23 DNA was cleaved with endonucleases SacII  
and HindIII, and a 1.0 kbp fragment containing the right  
25 border isolated and purified following agarose gel  
electrophoresis. Plasmid pUC18/neo(Bam) DNA was digested  
with endonucleases SacII and HindIII and the 4.0 kbp  
vector fragment isolated by agarose gel electrophoresis.  
The two fragments were mixed, incubated with T4 DNA  
30 ligase and transformed into E. coli strain HB101. The  
resulting plasmid, pCIB4 (shown in FIG. 23), contains  
the T-DNA right border and the plant-selectable marker  
for kanamycin resistance in a derivative of plasmid pUC18.

Next, a plasmid was constructed which contains both  
35 the T-DNA left and right borders, with the plant selectable

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1 kanamycin-resistance gene and the polylinker of pUC18  
between the borders (see FIG. 28). Plasmid pCIB4 DNA  
was digested with endonuclease HindIII, followed by  
treatment with the large fragment of DNA polymerase to  
5 create flush ends, followed by digestion with endonuclease  
EcoRI. The 2.6-kbp fragment containing the chimeric  
kanamycin-resistance gene and the right border of T-DNA  
was isolated by agarose gel electrophoresis. Plasmid  
pCIB5 DNA was digested with endonuclease AatII, treated  
10 with T4 DNA polymerase to create flush ends, then cleaved  
with endonuclease EcoRI. The larger vector fragment was  
purified by agarose gel electrophoresis, mixed with the  
pCIB4 fragment, incubated with T4 DNA ligase, and  
transformed into *E. coli* strain HB101. The resulting  
15 plasmid, pCIB2 (shown in FIG. 24) is a derivative of  
plasmid pBR322 containing the desired sequences between  
the two T-DNA borders.

The following steps complete construction of the  
vector pCIB10, and are shown in FIG. 25. Plasmid pCIB2  
20 DNA was digested with endonuclease EcoRV, and synthetic  
linkers containing BglII recognition sites are added as  
described above. After digestion with an excess of  
BglII endonuclease, the approximately 2.6-kbp fragment  
was isolated after agarose gel electrophoresis. Plasmid  
25 pRK252/Tn903/BglII, described above (see FIG. 20) was  
digested with endonuclease BglII and then treated with  
phosphatase to prevent recircularization. These two DNA  
fragments are mixed, incubated with T4 DNA ligase and  
transformed into *E. coli* strain HB101. The resulting  
30 plasmid is the completed vector, pCIB10.

Insertion of the chimeric protoxin gene into vector  
pCIB10 is by the steps shown in FIG. 26. Plasmid  
pBR322/btl4 DNA was digested with endonucleases PvuI and  
SalI, and then partially digested with endonuclease  
35 BamHI. A BamHI-SalI fragment approx. 4.2 kbp in size,

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1 containing the chimeric gene, was isolated following  
agarose gel electrophoresis, and mixed with plasmid  
pCIB10 DNA which had been digested with endonucleases  
BamHI and SalI. After incubation with T4 DNA ligase and  
5 transformation into E. Coli strain HB101, plasmid shown  
in FIG. 26 and contained the chimeric protoxin gene in  
the plasmid vector pCIB10.

In order to transfer plasmid pCIB10/19Sbt from E.  
coli HB101 to Agrobacterium, an intermediate E. coli  
10 host strain S17-1 was used. This strain, obtainable  
from Agrigenetics Research Corp., Boulder, Co. contains  
mobilization functions that transfer plasmid pCIB10  
directly to Agrobacterium via conjugation, thus avoiding  
the necessity to transform naked plasmid DNA directly  
15 into Agrobacterium (reference for strain S17-1 is Simon  
et al., "Molecular Genetics of the Bacteria-Plant  
Interaction", A Puhler, ed, Springer Verlag, Berlin,  
pages 98-106, 1983, incorporated herein by reference).  
First, plasmid pCIB10/19Sbt DNA is introduced into calcium  
20 chloride-treated S17-1 cells. Next, cultures of  
transformed S17-1 cells and Agrobacterium tumefaciens  
strain LBA4404 [Ooms et al., Gene, 14, 33-50 (1981)  
incorporated herein by reference] were mixed and mated  
on an N agar (Difco) plate overnight at room temperature.  
25 A loopful of the resulting bacteria are streaked onto AB  
minimal media; Chilton et al., Proc. Natl. Acad. Sci.  
USA, 77, 7347-7351 (1974), incorporated herein by  
reference, plated with 50ug/ml kanamycin and incubated  
at 28°C. Colonies were restreaked onto the same media,  
30 then restreaked onto NB agar plates. Slow-growing colonies  
were picked, restreaked onto AB minimal media with  
kanamycin and single colonies isolated. This procedure  
selects for Agrobacteria containing the pCIB10/19Sbt  
plasmid.

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1 Construction of a *Bacillus thuringiensis* protoxin  
chimeric gene with the CaMV 35S promoter was achieved by  
construction of a CaMV 35S Promoter Cassette Plasmid  
pCIB710 was constructed as shown in FIG. 27. This plasmid  
5 contained CaMV promoter and transcription termination  
sequences for the 35S RNA transcript [Covey, S.N.,  
Lomonossoff, G.P. and Hull, R., *Nucleic Acids Research*  
vol. 9, 6735-6747 (1981) incorporated herein by reference].  
A 1149-bp BglIII restriction fragment of CaMV DNA in Hohn  
10 et al., *Current Topics in Microbiology and Immunology*,  
96, 194-220 and Appendices A to G (1982) incorporated  
herein by reference] was isolated from plasmid pIV111  
(obtained from Dr. S. Howell Univ. California-San Diego;  
alternatively, the fragment can be isolated directly  
15 from CaMV DNA) by preparative agarose gel electrophoresis  
as described earlier and mixed with BamHI-cleaved plasmid  
pUC19 DNA, treated with T4 DNA ligase, and transformed  
into *E. coli*. The BamHI restriction site in the resulting  
plasmid has been destroyed by ligation of the BglIII  
20 cohesive ends to the BamHI cohesive ends. The resulting  
plasmid, called pUC19/35S, was then used in  
oligonucleotide-directed in-vitro mutagenesis to insert  
the BamHI recognition sequence GGATCC immediately  
following CaMV nucleotide 7483 in the Hohn reference.  
25 The resulting plasmid, pCIB710, contains the CaMV 35S  
promotor region and transcription termination region  
separated by a BamHI restriction site. DNA sequences  
inserted into this BamHI site will be expressed in plants  
by the CaMV transcription regulation sequences. pCIB710  
30 does not contain any ATG translation initiation codons  
between the start of transcription and the BamHI site.

Insertion of the CaMV 35S promoter/Terminator Cassette  
into pCIB10 occurred by the steps outlined in FIG. 28.  
Plasmids pCIB10 and pCIB710 DNAs were digested with  
35 EcoRI and SalI, mixed and ligated. The resulting plasmid,

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1     pCIB10/710 has the CaMV 35S promoter/terminator cassette  
inserted into the plant transformation vector pCIB10.  
The CaMV 35S sequences are between the T-DNA borders in  
pCIB10, and thus will be inserted into the plant genome  
5     in plant transformation.

Insertion of the *Bacillus thuringiensis* protoxin gene  
into pCIB10/710 occurred by the steps outlined in FIG.  
29. As a source of the protoxin gene, plasmid pCIB10/19Sbt  
was digested with BamHI and NcoI, and the 3.6-kb fragment  
10    containing the protoxin gene was isolated by preparative  
gel electrophoresis. The fragment was then mixed with  
synthetic NcoI-BamHI adapter with the sequence 5'-  
CATGGCCGGATCCGGC-3', then digested with BamHI. This  
step creates BamHI cohesive ends at both ends of the  
15    protoxin fragment. This fragment was then inserted into  
BamHI-cleaved pCIB10/710. The resulting plasmid,  
pCIB10/35Sbt, shown in FIG. 29, contains the protoxin  
gene between the CaMV 35S promoter and transcription  
termination sequences.

20    Transfer of the plasmid pCIB10/35Sbt into  
*Agrobacterium tumefaciens* strain LBA4404 was as described  
above.

Construction of a deleted *Bacillus thuringiensis*  
protoxin gene containing approximately 725 amino acids,  
25    and construction of a chimeric gene containing this  
deleted gene with the CaMV 35S promoter was made by  
removing the COOH-terminal portion of the gene by cleaving  
at the KpnI restriction endonuclease site at position  
2325 in the sequence shown in Formula 1. Plasmid  
30    pCIB10/35Sbt (FIG. 29) was digested with BamHI and KpnI,  
and the approximately 2.2-kbp BamHI/KpnI fragment  
containing the deleted protoxin gene isolated by  
preparative agarose gel electrophoresis. To convert the  
KpnI site at the 3' end to a BamHI site, the fragment  
35    was mixed with a KpnI/BamHI adapter oligonucleotide and

-44-

1 ligated. This fragment is then mixed with BamHI-cleaved  
pCIB10/710 (FIG. 28).

5 A deleted protoxin gene containing approximately  
645 amino acids was made by removing the COOH-terminal  
portion of the gene by cleaving at the BclI restriction  
endonuclease site at position 2090 in the sequence shown  
in Formula 1. Plasmid pCIB10/35Sbt (FIG. 29) was digested  
with BamHI and BclI, and the approximately 1.9-kbp  
BamHI/BclI fragment containing the deleted protoxin gene  
10 isolated by reparamative agarose gel electrophoresis.  
Since BclI creates a cohesive end compatible with BamHI,  
no further manipulation is required prior to ligating  
this fragment into BamHI-cleaved pCIB10/710 (FIG. 28).  
The resulting plasmid, which has the structure  
15 pCIB10/35Sbt(BclI) shown in FIG. 31 was selected on  
kanamycin.

The resulting transformants, designated  
pCIB10/35Sbt(KpnI) and shown in FIG. 30, contain the  
deleted protoxin gene of approximately 725 amino acids.  
20 These transformants are selected on kanamycin.

A deleted protoxin gene was made by introducing a  
BamHI cleavage site (GGATCC). This is done by cloning  
the BamHI fragment containing the protoxin sequence from  
pCIB10/35Sbt into mp18, and using standard oligonucleotide  
25 mutagenesis procedures described above. After mutagenesis,  
double-stranded replicative form DNA is prepared from the  
M13 clone, which is then digested with BamHI. The  
approximately 1.9-kbp fragment containing the deleted  
protoxin gene is inserted into BamHI-cleaved pCIB10/710.  
30 The resulting plasmid, which the structure  
pCIB10/35Sbt(607) shown in FIG. 32 is selected for on  
kanamycin.

The pCIB10/Sbt 607 was used. Transformation was  
accomplished as detailed in Example 7 with the change  
35 that the 1 ml aliquots were plated immediately on medium

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1 containing selective antibiotics. This selection medium  
contained kanamycin (50 ug/ml) or G418 (25 ug/ml).  
Expression of the NPT chimeric gene in both transformed  
plant tissue allows the selection of this tissue on either  
5 antibiotic.

In 2-4 weeks, transformed tissue became apparent on  
the selection plates. Plant material was selected on  
kanamycin or G418. Plant tissue (either individual  
embryos or callus) was then extracted with buffer and  
10 assayed for expression of the BT gene product by ELISA  
assay. The conditions of extraction are as follows:  
per 100mg of tissue, homogenize in 0.1 ml of extraction  
buffer containing 50 mM NaCO<sub>3</sub> (pH9.5), 0.05% Triton,  
0.05% Tween, 100mMNaCl, 10mM EDTA, 1mM leupeptine, and  
15 1mM PMSF. The leupeptine and PMSF are added immediately  
prior to use from 100x stock solutions. The tissue was  
ground with a motor driven pestle. After extraction, 2M  
Tris pH7 was added to adjust pH to 8.0-8.5 then centrifuged  
at 12,000 RPM in a Beckman microfuge 12 (10 minutes at  
20 4°C), and the supernatant saved for enzyme linked  
immunosorbent assay ("ELISA").

ELISA techniques as a general tool is described by  
M. F. Clark et al in Methods in Enzymology 118:742-766  
(1986), incorporated by reference.

25 An ELISA for the Bt toxin was developed using standard  
procedures and used to analyze transgenic plant material  
for expression of Bt sequences. For this procedure, an  
ELISA plate is pretreated with ethanol and affinity-  
purified rabbit anti-Bt antiserum (50 ul) at a  
30 concentration of 3 ug/ml in borate-buffered saline (see  
below) is added to the plate. This was allowed to incubate  
overnight at 4°C. Antiserum was produced in response to  
immunizing rabbits with gradient-purified Bt crystals  
[Ang, B.J. & Nickerson, K.W.; Appl. Environ. Microbiol.  
35 36: 625-626 (1978)], incorporated herein by reference,



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1 solubilized with sodium dodecyl sulfate and washed with  
 ELISA Wash Buffer (see below). It was then treated for  
 1 hour at room temperature with Blocking Buffer (see  
 below) washed with ELISA Wash Buffer. Plant extract was  
 5 added in an amount to give 50 ug of protein (this is  
 typically ca. 5 microliters of extract). Leaf extraction  
 buffer as protein is determined by the Bradford method  
 [Bradford, M., Anal. Biochem. 72:248 (1976) incorporated  
 herein by reference] using a commercially available kit  
 10 obtained from Bio-Rad, Richmond, California. If dilution  
 of the leaf extract is necessary, ELISA Diluent (see  
 below) is used. Allow this to incubate overnight at  
 4°C. After a wash with ELISA Wash Buffer, 50 ul affinity-  
 purified goat anti-Bt antiserum is added at a concentration  
 15 of 3 ug/ml protein in ELISA Diluent. This is allowed to  
 incubate for 1 hour at 37°C, then washed with ELISA Wash  
 Buffer. 50 ul rabbit anti-goat antibody bound to alkaline  
 phosphatase [commercially available from Sigma Chemicals,  
 St. Louis, Mo.] is diluted 1:500 in ELISA Diluent and  
 20 allowed to incubate for 1 hour at 37°C, then washed with  
 ELISA Wash Buffer. 50 microliters substrate [0.6 mg/ml  
 p-nitrophenyl phosphate in ELISA Substrate Buffer (see  
 below) are added and incubated for 30 minutes at room  
 temperature. Reaction is terminated by adding 50  
 25 microliters of 3 M NaOH. Absorbance is read at 405 nm  
 in modified ELISA reader [Hewlett Packard, Stanford,  
 Ca.].

Plant tissue transformed with the pCIB10/35SBt(BclI)  
 when assayed using this ELISA procedure showed a positive  
 30 reaction, indicating expression of the Bt gene.

EPBS (ELISA Phosphate Buffered Saline)

10 mM NaPhosphate:	$\text{Na}_2\text{HPO}_4$	4.68 grams/4 liters
	$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	0.976 grams/4 liters
35 140 mM NaCl	NaCl	32.7 grams/4 liters

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pH should be approximately 7.4

Borate Buffered Saline

100 mM Boric acid

25 mM Na Borate

75 mM NaCl

Adjust pH to 8.4-8.5 with HCl or NaOH as needed.

ELISA Blocking Buffer

In EPBS,

1% BSA

0.02% Na azide

ELISA Wash Buffer

10mM Tris-HCl pH 8.0

0.05% Tween 20

0.02% Na Azide

2.5M TRIS

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1     ELISA Diluent

In EPBS:

0.05% Tween 20

1% BSA

5     0.02% Na Azide

ELISA Substrate Buffer

In 500 mls,

48 ml Diethanolamine,

10    24.5 mg  $MgCl_2$ ;

adjust to pH 9.8 with HCl.

ELISA Substrate

15    15 mg p-nitrophenyl phosphate in 25 ml Substrate Buffer.

For bioassays, cell suspensions from antibiotic-resistant cell cultures obtained from transformations with these Agrobacteria were initiated. Suspensions were grown in medium supplemented with G418 (25mg/L), and subcultured into fresh antibiotic-containing medium on 7-10 day intervals. Samples of these cultures were then used in bioassays to test for toxicity to lepidopterous insects. Twenty ml aliquots of these cultures were allowed to settle (cell volume = 3-4ml), and resuspended in medium lacking antibiotics. Suspensions were then allowed to grow for an additional two days in this medium to deplete the cells of any residual antibiotic. Two circles of wet Whatman 2.3 cm filter paper were placed in the bottom of a 3/4 oz portion cup. A layer of transformed suspension culture cells 0.2 cm deep was placed onto the filter paper disk. A newly-hatched Manduca sexta or Heliothis virescens larva was placed into each portion cup. Controls were made up of larvae fed on non-transformed suspension culture cells. Discs were replenished on 2-day intervals or as needed.

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- 1     Manduca larvae generally require more plant material.  
The growth rate and mortality of the larvae feeding on  
transformed cells compared with the growth rate of larvae  
feeding on untransformed cells was scored after 5 days,  
5     and clearly affirmed the toxicity of the BT gene product  
in transformed cotton cells.

EXAMPLE 15

- 10     Heliothis virescens eggs laid on sheets of cheesecloth  
are obtained from the Tobacco Insect Control Laboratory  
at North Carolina State University, Raleigh, North  
Carolina. The cheesecloth sheets are transferred to a  
large covered glass beaker and incubated at 29 degrees C  
15     with wet paper towels to maintain humidity. The eggs  
hatched within three days. As soon as possible after  
hatching, the larvae (one larva per cup) are transferred  
to covered 3/4 oz. plastic cups. Each cup contains  
cotton leaf discs. Larvae are transferred using a fine  
20     bristle paint brush.

- Leaf discs one centimeter in diameter are punched  
from leaves of cotton plants and placed on a circle of  
wet filter paper in the cup with the larva. At least 6-  
10 leaf discs, representing both young and old leaves,  
25     are tested from each plant. Leaf discs are replaced at  
two-day intervals, or as necessary to feed the larvae.  
Growth rates [size or combined weight of all replica  
worms] and mortality of larvae feeding on leaves of  
transformed plants are compared with those of larva  
30     feeding on untransformed cotton leaves.

Larvae feeding on discs of cotton transformed with  
pCIB10/35SB5(BclI) show a decrease in growth rate and  
increase in mortality compared with controls.

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1        It was observed that a certain number of our  
regenerated plants (5-10%) appeared to have acquired  
genetically heritable phenotypic variations as a  
consequence of the process of regeneration. This variation  
5        is known as somaclonal variation. The following examples  
illustrate how somaclonal variation as a consequence of  
our regeneration procedure has been used to introduce  
commercially useful new traits into cotton varieties.

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EXAMPLE 16Cotton Regenerants Tolerant to Fungal Pathogens

      The procedure of Example 1 was followed, and  
regenerated cotton plants obtained of the variety SJ5  
15        and SJ4 were hardened and placed in the soil. These  
plants were self-pollinated and the seed, representing  
the F1 generation, collected.

      To obtain regenerants (somaclonal variants) more  
tolerant to Verticillium, the F1 generation was planted  
20        in a Verticillium infested field for progeny row analysis.  
Seed of the varieties SJ4 and SJ5 were planted in the  
field as controls. Somaclonal variants more tolerant  
than the parental varieties to the Verticillium fungus  
were identified in a few of the progeny rows (5%) by  
25        assessing overall plant vigor, yield, and the absence of  
foliar symptoms associated with the disease. FIG. 33  
shows the progeny rows of regenerants planted in a  
Verticillium infested field. FIG. 34 shows a Verticillium  
tolerant somaclonal variant of variety SJ4. This  
30        improvement in tolerance to the fungal pathogen was  
found to be genetically stable and passed on to subsequent  
generations.

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EXAMPLE 17Cotton Regenerants with altered growth habits

The procedure of Example 13 was followed except that, rather than planting in disease-infested soil, the F1 generation was planted in a cotton breeding nursery. The overall growth habit of the F1 regenerated progeny was compared to that of the control varieties. Somaclonal variants were identified which were more uniform in growth habit and shorter in stature than the parental variety. One SJ5 regenerant, identified in our trials as Phy 6, was 20% shorter in stature than the parental variety. This kind of growth habit is desirable in cotton grown under narrow row (30" row spacing) cultural conditions. These traits were found to be genetically stable and passed on to subsequent generations.

EXAMPLE 18

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Cotton regenerants with improved fiber traits

The procedure of Example 13 was followed except that the F1 progeny of regenerants were planted in a cotton breeding nursery and allowed to set fruit. When the bolls were mature, the cotton was harvested and subjected to an analysis of several fiber quality traits including length, uniformity, tensile strength, elasticity, and micronaire. Somaclonal variants were identified which were improved significantly over the parental variety in one or more of these traits. Representative data from F2 progeny (cell pollination of the F1) are included in the following Table 1. Values marked with an asterisk represent improvements in SJ5 regenerants which are statistically significant and have been found to breed true in subsequent generations.

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Table 1

Variety or strain	Length	Uniformity Index	<u>Fiber Properties</u>		
			Tensile Strength	Elasticity	Micronaire
SJ5	1.13	48.7	24.7	6.8	4.27
3SP16	1.27*	51.2	24.6	8.0*	4.10*
3SP20	1.28*	53.1*	23.1	7.6*	4.13*
5SP10	1.11	53.2*	25.7*	6.2	4.55
5SP17	1.18	51.7	26.7*	7.1	4.43

EXAMPLE 19

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Cotton regenerants with improved yield

The procedure of Example 13 was followed except that the F1 progeny of regenerants of the variety SJ4 were planted in replicated yield trials along with nonregenerated controls. One variant, which exhibited a more uniform growth habit and more vigorous growth habit, yielded 4% more cotton than the parental variety in the same trial. The data are given in Table 2 below.

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Table 2

<u>Variety or Strain</u>	<u><math>\bar{X}</math> Yield per plot (lb)</u>	<u><math>\bar{X}</math> Yield lbs/Acre</u>	<u>% Increase</u>
SJ4 Control	28.0	3049	
Phy 4	29.1	3169	4%*

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\*This difference was significant at the 95% confidence level.

A 4% increase in yield would represent a return of almost \$20 per acre to the average cotton grower in

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- 1 California, where over one million acres of cotton are grown annually.

EXAMPLE 20

- 5 Cotton Regenerants tolerant to a herbicide. (kanamycin)

Suspension cultures of the cotton variety B1644 were developed according to the method of Example 5. Suspension cultures were then plated onto an agar medium as described in Example 6, but supplemented with the herbicide (antibiotic) kanamycin (25mg/l). Most of the cells in the population died, but a few (1 to 5%) were tolerant and survived. These were selectively subcultured onto agar-solidified media supplemented with increasing concentrations of kanamycin, until the final concentration reached 50mg/l. Embryos were then developed from this callus, and those resistant embryos were germinated into kanamycin resistant plants.

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## FORMULA I

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      10      20      30      40      50      60
GTTAACACCC TGGGTCAAAA ATTGATATTT AGTAAATTA GTTGCACCTT GTGCATTTTT

      70      80      90     100     110     120
TCATAAGATG AGTCATATGT TTTAAATTGT AGTAATGAAA AACAGTATTA TATCATAATG

      130     140     150     160     170     180
AATTGGTATC TTAATAAAAG AGATGGAAGT AACTTATGGA TAACAATCCG AACATCAATG

      190     200     210     220     230     240
AATGCATTCC TTATAATTGT TTAAGTAACC CTGAAGTAGA AGTATTAGGT GGAGAAAGAA

      250     260     270     280     290     300
TAGAAACTGG TTACACCCCA ATCGATATTT CCTTGTGGCT AACGCAATTT ETTTGTAGTG

      310     320     330     340     350     360
AATTTGTTCC CGGTGCTGGA TTTGTGTTAG GACTAGTTGA TATAATATGG GGAATTTTTG

      370     380     390     400     410     420
GTCCCTCTCA ATGGGACGCA TTTCTGTAC AAATTGAACA GTTAATTAAE CAAAGAATAG

      430     440     450     460     470     480
AAGAATTCGC TAGGAACCAA GCCATTTCTA GATTAGAAGG ACTAAGCAAT CTTTATCANA

      490     500     510     520     530     540
TTTACGCAGA ATCTTTTAGA GAGTGGGAAG CAGATCCTAC TAATCCAGCA TTAAGAGAGG

      550     560     570     580     590     600
AGATGCGTAT TCAATTCAAT GACAISAAAC GTGCCCCCTAC AACCGCTATT CCTCTTTTTG

      610     620     630     640     650     660
CAGTTCAAAA TTATCAAGTT CCTCTTTTAT CAGTATATGT TCAAGCTGCA AATTTACATT

      670     680     690     700     710     720
TATCAGTTTT GAGAGATGTT TCAGTGTGTTG GACAAAGGTT GGGATTGAT GCGCGACTA

      730     740     750     760     770     780
TCAATAGTCG TTATAATGAT TTAAGTAGGC TTATTGGCAA CTATACAGAT CATGCTGTAC

      790     800     810     820     830     840
GCTGGTACAA TACGGGATTA GAGCGTGAT GGGGACCGGA TTCTAGAGAT TGGATAAGAT

      850     860     870     880     890     900
ATAATCAATT TAGAAGAGAA TTAACACTAA CTGTATTAGA TATCGTTTCT CTATTTCGA

      910     920     930     940     950     960
ACTATGATAG TAGAACGTAT CCAATTGGA CAGTTTCCCA ATTAACAAGA GAAATTTATA

      970     980     990    1000    1010    1020
CAAACCCAGT ATTAGAAAAT TTTGATGATA GTTTTCGAGG CTCGGCTCAG GGCATAGAGG

      1030    1040    1050    1060    1070    1080
GAAGTATTAG GAGTCCACAT TTGATGGATA TACTTAACAG TATAACCATC TATACGGATG

      1090    1100    1110    1120    1130    1140
CTCATAGAGG AGAATATTAT TGGTCAGGGC ATCAAATAAT GGCTTCTCCT GTAGGGTTTT

      1150    1160    1170    1180    1190    1200
CGGGGCCAGA ATTCACTTTT CCGCTATATG GAAGTATGGG AAATGCAGCT CCACAACAAC

      1210    1220    1230    1240    1250    1260
GAATTGTTGC TCAACTAGGT CAGGGCGTGT ATAGAACATT ATCGTCCACT TTATATAGAA

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1  
1270 1280 1290 1300 1310 1320  
GACCTTTTAA TATAAGGATA AATAATCAAC AACTATCTGT TCTTGACGGG ACAGAATTTG

5  
1330 1340 1350 1360 1370 1380  
CTTATGGAAC CTCCTCAAAT TTCCCATCCG CTGTATACAG AAAAAGCGGA ACGGTAGATT

10  
1390 1400 1410 1420 1430 1440  
CGCTGGATGA AATACGCCCA CAGAATAACA ACGTGCCACC TAGGCAAGGA TTTAGTCATC

15  
1450 1460 1470 1480 1490 1500  
GATTAAGCCA TGTTCGAATG TTTCSTTCAG GCTTTAGTAA TAGTAGTGA AGTATAATAA

20  
1510 1520 1530 1540 1550 1560  
GAGCTCCTAT GTTCTCTTBG ATACATCGTA GTGCTGAATT TAATAATATA ATTCCTTCAT

25  
1570 1580 1590 1600 1610 1620  
CACAAATTAC ACAATACCT TTAACAAAAT CTACTAATCT TGGCTCTGGA ACTTCTGTCC

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1630 1640 1650 1660 1670 1680  
TTAAAGGACC AGGATTTACA GGAGGAGATA TTCTTCGAAG AACTTCACCT GGCCAGATT

35  
1690 1700 1710 1720 1730 1740  
CAACCTTAAG AGTAAATATT ACTGCACCAT TATCACAAG ATATCGGGTA AGAATTCGCT

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1750 1760 1770 1780 1790 1800  
ACGCTTCTAC CACAAATTTA CAATTCATA CATCAATTGA CGGAAGACCT ATTAATCAGG

45  
1810 1820 1830 1840 1850 1860  
GGAATTTTTT AGCAACTATG AGTAGTGGA GTAATTTACA GTCCGGAGCG TTTAGGACTG

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1870 1880 1890 1900 1910 1920  
TAGGTTTTAC TACTCCSTTT AACTTTTCAA ATGGATCAAG TGTATTACG TTAAGTGCTC

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1930 1940 1950 1960 1970 1980  
ATGTCTTCAA TTCAGGCAAT GAAGTTTATA TAGATCGAAT TGAATTTGTT CCGGCAGAAG

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1990 2000 2010 2020 2030 2040  
TAACCTTTGA GGCAGAAATAT GATTTAGAAA GAGCACAAAA GGCGGTGAAT GAGCTGTTTA

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2050 2060 2070 2080 2090 2100  
CTTCTTCCAA TCAAAATCGG TTAACAAACAG ATGTGACGGA TTATCATATT GATCAAGTAT

70  
2110 2120 2130 2140 2150 2160  
CCAATTTAGT TGAGTGTTTA TCTGATGAAT TTTGTCTGGA TGAAAAAAA GAATTGTCCG

75  
2170 2180 2190 2200 2210 2220  
AGAAAGTCAA ACATGCGAAG CGACTTAATG ATGAGCGGAA TTTACTTCAA GATCCAAACT

80  
2230 2240 2250 2260 2270 2280  
YTAGAGGGAT CAATAGAGAA CTAGACCGTG GCTGGAGAGG AAGTACGGAT ATTACCATCC

85  
2290 2300 2310 2320 2330 2340  
AAGGAGGCGA TGACGTATTC AAAGAGAAAT ACSTTACGCT ATTGGGTACC TTTGATGAGT

90  
2350 2360 2370 2380 2390 2400  
GCTATCCAAC GTATTATAT CAAAAATAG ATGAGTCGAA ATTAAAGCC TATACCCGTT

95  
2410 2420 2430 2440 2450 2460  
ACCAATTAAG AGGGTATATC GAAGATAGTC AAGACTTAGA AATCTATTIA ATTCCGTACA

100  
2470 2480 2490 2500 2510 2520  
ATGCCAAACA CGAAACAGTA AATGTGCCAG GTACGGGTTT CTTATGGCCG CTTTCAGCCC

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2530 2540 2550 2560 2570 2580  
 CAAGTCCAAT CCGAAAATGT GCCCATCATT CCCATCATT CTCCTTGGAC ATTGATGTTG  
 2590 2600 2610 2620 2630 2640  
 GATGTACAGA CTTAAATGAG GACTTAGGTG TATGGGTGAT ATTCAAGATT AAGACGCAAG  
 2650 2660 2670 2680 2690 2700  
 ATGGCCATGC AAGACTAGGA AATCTAGAAT TTCTCGAAGA GAAACCATTG GTAGGAGAAAG  
 2710 2720 2730 2740 2750 2760  
 CACTAGCTCG TGTGAAGAAGA GCGGAGAAAA AATGGAGAGA CAACCGTGAA AAATTGGAAT  
 2770 2780 2790 2800 2810 2820  
 GGGAAACAAA TATTGTTTAT AAAGAGGCAA AAGAATCTGT AGATGCTTTA TTTGTAAACT  
 2830 2840 2850 2860 2870 2880  
 CTCAATATGA TAGATTACAA GCGGATACCA ACATCGCGAT GATTCATGCG GCAGATAAAC  
 2890 2900 2910 2920 2930 2940  
 GCGTTCATAG CATTGAGAGAA GCTTATCTGC CTGAGCTGTC TGTGATTECG GGTGTCAATG  
 2950 2960 2970 2980 2990 3000  
 CGGCTATTTT TGAAGAATTA GAAGGCGSTA TTTTCACTGC ATTCTCCCTA TATGATGCGA  
 3010 3020 3030 3040 3050 3060  
 GAAATGTCAT TAAAAATGGT GATTTTAATA ATGGCTTATC CTGCTGGAAC GTGAAAGGSC  
 3070 3080 3090 3100 3110 3120  
 ATGTAGATGT ABAAGAACAA AACAAACCACC GTTCGGTCTT TGTGTTCGCG GAATGGGAAG  
 3130 3140 3150 3160 3170 3180  
 CAGAAGTGTG ACAAGAAGTT CGTGTCTGTC CCGGTCTGTG CTATATCCTT CGTGTCAACG  
 3190 3200 3210 3220 3230 3240  
 CGTACAAGGA GGGATATGGA GAAGGTTGCG TAACCATTCA TGAGATCGAG AACAAATACAG  
 3250 3260 3270 3280 3290 3300  
 ACGAACTGAA GTTTAGCAAC TGTGTAGAAG AGGAAGTATA TCCAAACAAC ACGGTAACGT  
 3310 3320 3330 3340 3350 3360  
 GTAATGATTA TACTGCBACT CAAGAAGBAAT ATBAGGGTAC GTACACTTCT CGTAATCGAG  
 3370 3380 3390 3400 3410 3420  
 GATATGACGG AGCCTATGAA AGCAATTCTT CTGTACCAGC TGATTATGCA TCAGCCTATG  
 3430 3440 3450 3460 3470 3480  
 AAGAAAAAGC ATATACAGAT GGACGAAGAG ACAATCCTTG TGAATCTAAC AGAGGATATG  
 3490 3500 3510 3520 3530 3540  
 GGGATTACAC ACCACTACCA GCTGGCTATG TGACAAAAGA ATTAGAGTAC TTCCCAAGAA  
 3550 3560 3570 3580 3590 3600  
 CCGATAAGGT ATGGATTGAG ATCGGAGAAA CGGAAGGAAC ATTCAACGTG GACAGCGTGG  
 3610 3620 3630 3640 3650 3660  
 AATTACTTCT TATGGAGGAA TAATATATGC TTTATAATGT AAGGTGTGCA AATAAAGAA  
 3670 3680 3690 3700 3710 3720  
 GATTACTBAC TTGTATTGAC AGATAAATAA GBAATTTTTT ATATGAATAA AAAACGGGCA  
 3730 3740 3750 3760 3770 3780  
 TCACTETTAA AAGAATGATG TCCGTTTTTT GATGATTTA ACGAGTGATA TTTAAATGTT

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3790 3800 3810 3820 3830 3840  
TTTTTGCBA AGGCTTTACT TAACGGGGTA CCGCCACATG CCCATCAACT TAAGAATTTG

3850 3860 3870 3880 3890 3900  
CACTACCCCG AAGTGTCAAA AAACGTTATT CTTTCTAAAA AGCTAGCTAG AAAGGATGAC

3910 3920 3930 3940 3950 3960  
ATTTTTTATG AATCTTTCAA TTCAAGATGA ATTACAACATA TTTTCTGAAG AGCTGTATCG

3970 3980 3990 4000 4010 4020  
TCATTTAACC CTTTCTCTTT TGGAGAAGACT CGCTAAAGAA TTAGGTTTTG TAAAAAGAAA

4030 4040 4050 4060 4070 4080  
ACGAAAGTTT TCAGGAATG AATTAGCTAC CATATGTATC TGGGGCAGTC AACGTACAGC

4090 4100 4110 4120 4130 4140  
GAGTBATTCT CTCGTTGAC TATGCAATCA ATTACACGCC GCCACAGCAC TCTTATGAGT

4150 4160 4170 4180 4190 4200  
CCAGAAGGAC TCAATAAACB CTTTGATAAA AAAGCGGTTG AATTTTGGAA ATATATTTTT

4210 4220 4230 4240 4250 4260  
TCTGCATTAT GGAAAGTAA ACTTTGTAAA ACATCAGCCA TTTCAAGTGC ASCACTCAGC

4270 4280 4290 4300 4310 4320  
TATTTTCAAC GAATCCGTAT TTTAGATCCG ACGATTTTCC AAGTACCGAA ACATTTAGCA

4330 4340 4350 4360  
CATGTATATC CTGGGTCAGG TGCTTGTGCA CAACTGCAG

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WHAT IS CLAIMED IS:

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1. A method for the regeneration of a cotton plant from somatic cells which comprises steps of:

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a) providing a cotton explant;

b) culturing the explant in a first callus growth medium for a period of time sufficient for undifferentiated callus to develop from the explant;

c) transferring the callus to a second callus growth medium;

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d) culturing the callus in the second callus growth medium for a period of time sufficient to allow development of embryogenic callus;

e) transferring the embryogenic callus to a plant germination medium; and

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f) culturing the embryogenic callus on the plant germination medium for a period of time sufficient to develop a plantlet from the embryogenic callus.

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2. The method as claimed in claim 1, in which the cotton seedling is developed by:

a) sterilizing the seed in a first sterilizing solution;

b) rinsing the seed in sterile water;

c) sterilizing the seed in a second sterilizing solution;

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f) reusing the second sterilization medium from the seed with sterile water;

g) transferring the seed to a seed germination medium; and

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h) growing the seed in the seed germination medium in the dark for a period of time sufficient to produce a seedling; and

i) excising the explant from the seedling.

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1           3.    The method as defined in claim 2 in which the  
first sterilizing solution is an aqueous solution  
containing about 95% by volume ethanol and the second  
sterilizing solution is an aqueous solution containing  
5    about 15% by weight sodium hypochlorite.

          4.    The method as claimed in claim 1 wherein the  
explant is selected from the group consisting of hypocotyl,  
cotyledon and mixtures thereof, and immature zygotic  
10   embryos.

          5.    The method as claimed in claim 2 wherein the  
explant is selected from the group consisting of hypocotyl,  
cotyledon and mixtures thereof.

15           6.    The method as claimed in claim 3 wherein the  
explant is selected from the group consisting of hypocotyl,  
cotyledon and mixtures thereof.

20           7.    A method as claimed in claim 2 in which seed  
germination medium is a basal agar medium and growth  
prior to explant removal is up to about 4 weeks.

          8.    The method as claimed in claim 1 in which the  
25   embryogenic callus are developed from the explant by  
growth in light-dark cycle of about 16 hours of light  
and about 8 hours of darkness at a temperature from  
about 25 to about 35°C.

30           9.    The method as claimed in claim 8 in which the  
light intensity during the hours of light is about 2,000  
to about 4,000 lux.

          10.   A method as claimed in claim 8 in which the light  
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1 intensity during the hours of light is about 3,000 to about  
4,000 lux.

5 11. A method as claimed in claim 1 in which callus  
are grown in the first callus growth medium containing  
glucose during a period of phenolic secretions with  
medium changed at least every 10 days.

10 12. The method as claimed in claim 11 wherein the  
explant is transferred to a fresh first callus growth  
medium at about 3 to about 4 weeks.

15 13. A method as claimed in claim 1 in which the first  
callus growth medium is a Murashige and Skoog medium  
comprising glucose.

20 14. A method as claimed in claim 1 in which the  
second callus growth medium is Murashige and Skoog medium  
comprising sucrose and from about 1 to about 10 mg/l  
naphthaleneacetic acid.

25 15. A method as claimed in claim 13 in which the  
second callus growth medium is Murashige and Skoog medium  
comprising sucrose and from about 1 to about 10 mg/l  
naphthaleneacetic acid.

30 16. The method as claimed in claim 1 in which the  
plant germination in medium is a Beasley and Ting's  
medium, rich in a source of nitrogen.

35 17. The method as claimed in claim 15 in which the  
step of the plant germination in medium is a Beasley and  
Ting's medium, rich in a source of nitrogen.

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1           18. The method as claimed in claim 1 further  
including the steps of transferring the plantlets to  
soil under condition of high humidity for a time sufficient  
for the plantlet to mature to enable transfer to a hot  
5 house or field for growth to final maturity.

19. A method for the regeneration of a cotton  
plant from somatic cells which comprises the steps of:

10           a) providing a cotton explant select from  
the group consisting hypocotyl, cotyledon and mixtures  
thereof from a cotton seeding and immature embryos;

15           b) culturing the explant in a first callus  
growth medium which is a full or half-strength Murashige  
and Skoog growth medium supplemented with thiamine  
hydrochloride, naphthaleneacetic acid and kinetin and  
inositol at a temperature of from about 25 to about 35°C  
under a day-light cycle of about 16 hours light at a  
light intensity of about 2,000 to about 4,000 lux and  
about 8 hours of darkness for a period of time sufficient  
20 for undifferentiated callus to form from the explant;

25           c) transferring the callus from the first  
callus growth medium to a second callus growth medium  
which is a full or half-strength Murashige and Skoog  
growth medium comprising sucrose and from about 1 to  
about 10 mg/l of naphthaleneacetic acid and culturing  
the callus at a temperature from about 25 to 35°C under  
a daylight cycle of about 16 hours light at a light  
intensity of about 2,000 to about 4,000 lux and about 8  
hours dark for a time sufficient to form yellow to white  
30 embryogenic callus.

          d) further subculturing the embryogenic callus  
to develop to callus containing somatic embryos;

          e) transferring somatic embryos to an embryo  
germination medium rich in a source of nitrogen and  
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1 growing the embryos to plantlets sufficiently developed  
for transfer to soil.

5 20. A method as claimed in claim 19 in which the  
first callus growth medium is a Murashige and Skoog  
growth medium containing about 0.4 mg/l thiamine  
hydrochloride, about 30 g/l sucrose, about 2 mg/l  
naphthaleneacetic acid about 1 mg/l kinetin and about 100  
mg/l inositol.

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21. A method as claimed in claim 19 in which the  
second callus growth medium contains from about 1 to  
about 5 mg/l naphthaleneacetic acid and from 0 to about  
1 mg/l cytokinin.

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22. A method as claimed in claim 20 in which the  
second callus growth medium contains from about 1 to  
about 5 mg/l naphthalene acetic acid and from 0 to about  
1 mg/l cytokinin.

20

23. A method as claimed in claim 19 in which the  
embryo germination medium is a Beasley and Ting's medium  
containing up to about 500 mg/l casein hydrolysate and up  
to about 1200 mg/l ammonium nitrate.

25

24. A method as claimed in claim 24 in which the  
embryo germination medium contains a source of ammonium.

30

25. A method as claimed in claim 22 in which the  
embryo germination medium is a Beasley and Ting's medium  
containing up to about 500 mg/l casein hydrolysate and  
up to about 1200 mg/l ammonium nitrate.

35

26. A method as claimed in claim 19 in which the  
first callus growth media during a period of phenol

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1       secretion from the callus is changed within about each  
ten days until phenol secretion stops following which  
sucrose is included in the callus growth medium.

5               27. A method as claimed in claim 25 in which the  
first callus growth media is during a period of phenol  
secretion from the callus changed within about each ten  
days until phenol secretion stops following which sucrose  
is included in the callus growth medium.

10

28. A method for the regeneration of cotton plants  
from somatic cells, comprising the steps of:

15               (a) sterilizing at least one cotton seed in a  
solution containing 95% by volume ethanol for a period  
of approximately 2-3 minutes;

              (b) rinsing the seed in sterile water;

20               (c) soaking the seed in a solution of sodium  
hypochlorite containing about 15% by weight sodium  
hypochlorite for a period of from about 15 to about 20  
minutes;

              (d) rinsing the seed in sterile water;

25               (e) germinating the seed in a dark environment  
on modified basal agar medium of Whites or half strength  
Murashige and Skoog medium for a period up to about  
fourteen days to produce a seedling;

              (f) excising segments selected from the  
hypocotyl, cotyledon or mixtures thereof from the seedling;

30               (g) culturing the excised segments on a  
Murashiga-Skoog medium supplemented with about 0.4 mg/l  
thiamine hydrochloride, about 30 g/l glucose, about 2  
mg/l naphthaleneacetic acid, about 1 mg/l kinetin and  
about 100 mg/l inositol for a period of approximately  
three to four weeks in an environment of 30°C under a  
light-dark cycle of 16 hours of light and about 8 hours

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1 of dark, at about 3,000 to 4,000 lux light intensity during  
the hours of light, to produce callus;

(h) transferring the callus onto Murashige and  
Skoog medium comprising sucrose and about 2 mg/liter  
5 naphthaleneacetic acid and about 1 mg/liter cytokinin;

(i) culturing the callus over a period of  
about three to four months to produce embryos;

(j) transferring the embryos to Beasley &  
Ting's medium comprising about 500 mg/liter casein  
10 hydrolysate, and a source of nitrogen and culturing the  
embryos for a period of about 2 to about 3 weeks, to  
produce plantlets; and

(k) transferring the plantlets to soil and  
incubating the plantlets in high humidity from plants.

15

27. The method as defined in claim 28 including  
the steps of:

(a) self-pollinating the plants so as to  
produce seeds; and

20 (b) germinating the seeds to produce seedlings.

30. A method for the regeneration of a cotton  
plant from somatic cells which comprises the steps of:

a) culturing a cotton explant by tissue culture  
25 on a callus growth medium for a period of time sufficient  
to develop embryogenic callus;

b) subdividing and suspending embryogenic callus  
in a second callus growth medium and growing said callus  
to form embryogenic clumps of at least 600 microns in size;

30 c) filtering out embryogenic clumps of a size  
greater than 600 microns;

d) growing the embryogenic clumps of a size greater  
than 600 microns in a plant germination medium for a  
period of time sufficient to develop plantlets from the  
35 clumps.

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1

31. A method as claimed in claim 30 in which the clump of a size less than 600 microns are resuspended in fresh second callus growth medium for further growth of embryogenic clumps.

5

32. A method as claimed in claim 30 in which the explant is obtained by:

a) sterilizing the seed in a first-sterilized solution;

10

b) rinsing the seed with sterile water;

c) sterilizing the seed in a second sterilizing solution;

15

d) rinsing the second sterilization medium from the seed with sterile water;

e) transferring the sterilized seed to a seed germination medium; and

20

f) culturing the seed in the seed germination medium in the dark for a period of time sufficient to form cotton seedling; and

g) excising the explant from the cotton seedling.

25

33. The method as claimed in claim 31 in which the explant is obtained by:

a) sterilizing the seed in a first sterilizing solution;

b) rinsing the seed with sterile water;

30

c) sterilizing the seed in a second sterilizing solution;

d) rinsing the second sterilization medium from the seed with water;

e) transferring the sterilized seed to a seed germination medium; and

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1                   f)     culturing the seed in the seed germination  
medium in the dark for a period of time sufficient to  
form cotton seedling; and

5                   g)     excising the explant from the cotton  
seedling.

34. The method of claim 33 in which the first  
sterilizing solution is an aqueous solution containing  
about 95% by volume ethanol; and the second sterilizing  
10 solution is an aqueous solution containing about 15% by  
weight sodium hypochlorite.

35. The method as claimed in claim 34, wherein the  
explant includes at least a portion of seedling parts  
15 selected from the hypocotyl, cotyledon and the mixtures  
thereof.

36. A method as claimed in claim 35 in which seed  
germination medium is a basic agar medium and growth  
20 prior to explant removal is for a period of up to about  
14 days.

37. A method as claimed in claim 30 in which clumps  
greater than about 800 microns are removed from suspension  
25 for plant growth.

38. A method as claimed in claim 30 in which the  
suspension culture at the beginning of growth contains from  
750 to about 1000 mg of callus parts per 8 ml second  
30 embryo growth medium.

39. A method as claimed in claim 31 in which the  
suspension culture at the beginning of growth is from  
750 to about 1000 mg of callus parts per 8 ml second  
35 embryo growth medium.

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40. The method as claimed in claim 30 in which the embryogenic callus are grown in the suspension in light-dark cycle of about 16 hours of light and about 8 hours of darkness at a temperature from about 25 to about 35°C.

5

41. The method as claimed in claim 31 in which the embryogenic callus are grown in the suspension in light-dark cycle of about 16 hours of light and about 8 hours of darkness at a temperature from about 25 to about 35°C.

10

42. The method as claimed in claim 40 in which the light intensity during the hours of light is about 2,000 to about 4,000 lux.

15

43. A method as claimed in claim 41 in which the light intensity during the hours of light is about 3,000 to about 4,000 lux.

20

44. A method as claimed in claim 30 in which the second callus growth medium is Murashige and Skoog medium containing naphthaleneacetic acid.

25

45. A method as claimed in claim 31 in which the second callus growth medium is Murashige and Skoog medium containing naphthaleneacetic acid.

30

46. The method as claimed in claim 30 in which the step of the plant germination in medium is a Beasley and Ting's medium, rich in a source of nitrogen.

35

47. The method as claimed in claim 30 in which the step of the plant germination in medium is a Beasley and Ting's medium, comprising casein hydrolysate and a source of ammonium.

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1

48. The method as claimed in claim 30 further including the steps of:

5 a) transferring the plantlets to soil under condition of high humidity for a time sufficient to mature to enable transfer to a hot house then field for growth to final maturity.

10 49. A method for the regeneration of a cotton plant from somatic cells which comprises the steps of:

a) providing an explant selected from the group consisting of hypocotyl, cotyledon and mixtures thereof derived from a cotton seedling and immature embryos;

15 b) culturing the explant in a first callus growth medium which is a Murashige and Skoog growth medium supplemented with thiamine hydrochloride, glucose, naphthaleneacetic, kinetin and inositol, at a temperature of from about 25 to about 35°C under a daylight cycle of  
20 about 16 hours light at a light intensity of about 2,000 to about 4,000 lux and about 8 hours of darkness for a period of time sufficient for undifferentiated callus to form from the explant;

25 c) transferring the callus from the first callus growth medium to a second callus growth medium which is Murashige and Skoog growth medium containing sucrose and from about 1 to about 10 mg/l of naphthaleneacetic acid and culturing the callus at a temperature from about 25 to 35°C under a daylight cycle  
30 of about 16 hours light at a light intensity of about 2,000 to about 4,000 lux and about 8 hours dark for a time sufficient to develop embryogenic callus;

d) suspending parts of the embryogenic callus in fresh second callus growth medium at a concentration  
35 from about 750 about 100 mg of callus parts per 8 ml second

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1 embryo growth medium and allowing the embryo to grow for  
a time sufficient to develop embryogenic clumps of a  
size greater than about 800 microns;

5 e) separating the embryogenic clumps greater than  
about 800 microns from clumps less than about 800 microns;

f) transferring the embryogenic clumps of a size  
greater than 800 microns to a germination medium rich in  
a source of nitrogen and growing the embryos to plantlets.

10 50. A method as claimed in claim 49 in which the  
embryogenic clumps less than 800 microns are resuspended  
in fresh second callus growth medium as per step d) and  
steps e) and f) repeated.

15 51. A method as claimed in claim 49 in which the  
first callus growth medium is a Murashige and Skoog  
growth medium containing about 0.4 mg/l thiamine  
hydrochloride, about 30 g/l glucose, about 2 mg/l  
naphthaleneacetic acid, about 1 mg/l kinetin and about  
20 100 mg/l inositol.

25 52. A method as claimed in claim 49 in which the  
second embryo growth media comprises sucrose and from 1  
to about 5 mg/l naphthaleneacetic acid and from 0 to about  
1 mg/l cytokinin.

30 53. A method as claimed in claim 50 in which the  
second embryo growth medium contains from 1 to about 5 mg/l  
naphthaleneacetic acid and from 0 to about 1 mg/l  
cytokinin.

35 54. A method as claimed in claim 51 in which the  
second embryo grow media contains from 1 to about 5 mg/l  
naphthaleneacetic acid and from 0 to about 1 mg/l  
cytokinin.



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55. A method as claimed in claim 49 in which the embryo germination medium is a Beasley and Ting's medium containing up to about 500 mg/l casein hydrolysate.

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1           56. A method as claimed in claim 50 in which the embryo germination medium is a Beasley and Ting's medium containing up to about 500 mg/l casein hydrolysate.

5           57. A method as claimed in claim 55 in which the embryo germination medium is a Beasley and Ting's medium containing up to about 500 mg/l casein hydrolysate.

          58. A method for transforming cotton which comprises:  
10           a) contacting a cotton explant with a Agrobacterium vector containing an expressible gene code foreign to cotton for a time sufficient to transfer the gene to the cells of explant;  
          b) incubating the explant in a callus growth  
15           medium for about 15 to about 200 hours at a temperature from about 25 to about 35°C under a cycle of 16 hours light and 8 hours dark.  
          c) contacting the incubated explants with a callus growth media containing an antibiotic which is  
20           toxic to the Agrobacterium for a time sufficient to kill the Agrobacterium;  
          d) culturing the explant free of the Agrobacterium in the callus growth media; and  
          e) selecting transformed callus from  
25           untransformed callus.

          59. A method claimed in claim 58 in which the supplemented callus growth medium is a Murashige and Skoog medium supplemented with about 1 to about 10 mg/l  
30           naphthaleneacetic acid.

          60. A method as claimed in claim 58 in which the antibiotic toxic to the Agrobacterium is cefotaxime.

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1           61. A method for transforming cotton which  
comprises;

5           a) contacting for a period of from about 1  
minute to about 24 hours, segments of cotton seedling  
explant selected from hypocotyl, cotyledon and mixtures  
thereof with an Agrobacterium vector containing a gene code  
which includes resistance to an antibiotic;

10          b) transferring the explants to a callus growth  
medium which is a Murashige and Skoog medium supplemented  
with about 1 to about 10 mg/l naphthaleneacetic acid for  
a period of from about 15 to about 200 hours at a  
temperature of from about 25 to about 35°C under a cycle  
of about 16 hours light and 8 hours dark to develop callus  
from the explants;

15          c) transferring the callus to a fresh callus  
growth medium containing cefotaxime in a concentration  
sufficient to kill off Agrobacterium;

          d) culturing the callus on fresh first callus  
growth medium; and

20          e) contacting callus with fresh callus growth  
medium additionally containing cefotaxime to kill off  
residual Agrobacterium and an antibiotic to which the  
transformed callus is insensitive to select callus  
resistant to the antibiotic.

25           62. A method as claimed in claim 61 in which the  
transformed callus prior to contact with the callus growth  
medium containing cefotaxime is rinsed in callus growth  
medium free of cefotaxime.

30           63. A method as claimed in claim 61 in which the  
antibiotic is kanamycin.

35           64. A method as claimed in claim 62 in which the  
antibiotic is kanamycin.

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65. A method of producing transformed embryogenic cotton callus from cotton cells undergoing suspension culture in a callus growth medium which comprises after a suspension subculture growth cycle;

5

a) recovering cells and any formed embryogenic callus from the callus growth medium;

10

b) resuspending the recovered cells and callus in a callus growth medium containing an Agrobacterium vector including an expressible gene sequence foreign to cotton while maintaining suspension growth conditions for period of time sufficient to transform the cotton cells;

15

c) recovering the suspended cells and callus from the callus growth medium containing the Agrobacterium;

d) treating the transformed cells and callus with an antibiotic which is toxic to the Agrobacterium to kill off the Agrobacterium;

20

e) selecting the transformed cotton cells and callus from untransformed cotton cells and embryogenic callus cells; and

f) filtering a suspension of the cells to remove embryogenic callus greater than about 600 microns.

25

66. A method as claimed in claim 65 in which the transformed cells and callus are treated with the antibiotic prior to filtering the suspension.

30

67. A method as claimed in claim 65 in which the transformed cells and callus are selected prior to filtration of the suspension.

35

68. A method as claimed in claim 66 in which the transformed cells and callus are selected prior to filtration of the suspension.

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1           69. A method as claimed in claim 65 in which the transformed cells and embryogenic callus are separately treated with the antibiotic following filtration of the suspension.

5

70. A method as claimed in claim 65 in which the transformed cells and embryogenic callus are selected following filtration of the suspension.

10

71. A method as claimed in claim 69 in which the transformed cells and embryogenic callus are selected following filtration of the suspension.

15

72. A method as claimed in claim 65 in which the antibiotic toxic to the Agrobacterium is cefotaxime.

73. A method as claimed in claim 66 in which the antibiotic toxic to the Agrobacterium is cefotaxime.

20

74. A method as claimed in claim 69 in which the antibiotic toxic to the Agrobacterium is cefotaxime.

25

75. A method of producing embryogenic cotton callus from cotton cells undergoing suspension culture in a callus growth medium which comprises after a suspension subculture growth cycle of from about 7 to about 14 days;

a) recovering cells from the callus growth medium;

30

b) resuspending the cells in a callus growth medium containing an Agrobacterium vector including an expressible gene sequence foreign to cotton while maintaining suspension growth conditions for period of time sufficient to transform the suspended cells;

35

c) recovering the cells from the callus growth medium containing the Agrobacterium;

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- 1                   d) further subculturing the cells in fresh  
callus growth medium free of the Agrobacterium;  
                  e) filtering the suspension to remove  
embryogenic callus greater than about 600 microns;  
5                   f) resuspending the residual cells in a callus  
growth medium containing an antibiotic which is toxic to  
the Agrobacterium;  
                  g) treating the embryogenic callus with an  
antibiotic which is toxic to the Agrobacterium; and  
10                  h) selecting transformed cells and embryogenic  
callus from untransformed cells and embryogenic callus.

15                  76. A method as claimed in claim 75 in which the  
antibiotic is kanamycin.

20                  77. A method of produced transformed cotton callus  
from cotton cells undergoing suspension culture in a  
callus growth medium which comprises after a suspension  
subculture growth cycle of from about 7 to about 14 days;

- 20                  a) recovering cells from the callus growth  
medium;  
                  b) resuspending the cells in a callus growth  
medium containing an Agrobacterium vector including an  
antibiotic resistant gene sequence while maintaining  
25                  suspension growth conditions for period of time sufficient  
to transform the suspended cells;  
                  c) recovering the cells from the callus  
growth medium containing the Agrobacterium;  
                  d) washing the cells in fresh callus growth  
30                  medium free of the Agrobacterium;  
                  e) further subculturing the cells in fresh  
callus growth medium free of the Agrobacterium;  
                  f) filtering the suspension to remove  
embryogenic callus greater than about 600 microns;

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1           g) resuspending the residual cells in a  
callus growth medium containing cefotaxime; and

          h) contacting suspended residual cells and  
removed embryogenic callus with an antibiotic to which  
5       the transformed cells are insensitive to select the  
transformed cells.

78. A method as claimed in claim 77 in which the  
antibiotic is kanamycin.

10       79. Cotton plants transformed to have resistance  
to antibiotics normally inhibitory to cotton plant cell  
growth.

15       80. Cotton plant regenerants exhibiting increased  
tolerance to verticillium wilt.

20       81. Cotton regenerants exhibiting improved fiber  
quality traits of at least greater fiber length, tensile  
strength, elasticity on lower micronaire as compared to  
the parental variety.

25       82. Cotton regenerants exhibiting herbicide  
tolerance.

83. Cotton regenerants exhibiting increased yield  
as compared to the parental variety.

30       84. Cotton regenerants exhibiting increased tolerance  
to fungal pathogens.

35       85. A vector for conferring antibiotic resistance  
to a cotton plant which comprises two T-DNA right border  
sequences from A. Tumefaciens capable of integration  
with the plant genome flanking a chimeric gene capable

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1 of expression in cotton and of conferring resistance to  
the antibiotics Kanamycin and G418.

5 86. A vector as claimed in claim 85 in which the  
chimeric gene comprises in sequence a napoline synthetase  
promoter, a neomycin phosphotransferase II coding region  
from Tn5 and the terminator from the herpes simplex  
virus thimidine kinase gene.

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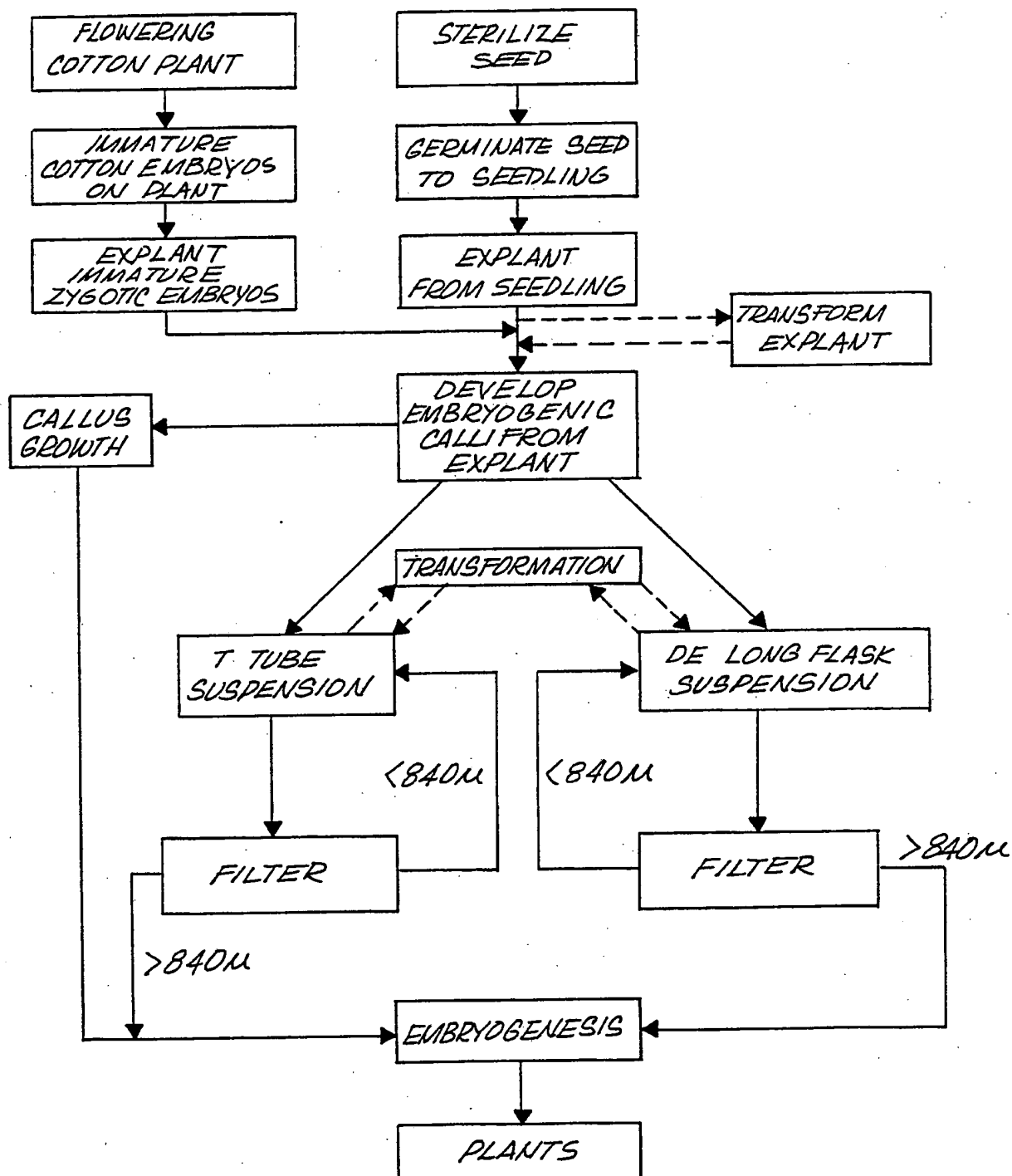
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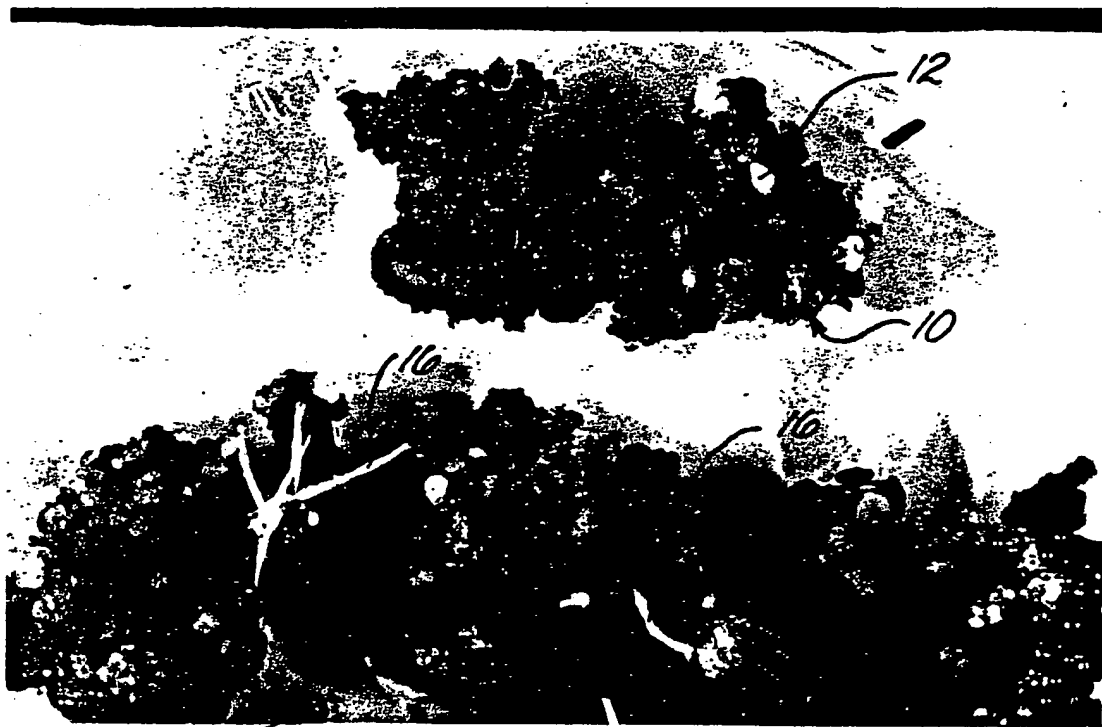
1 / 29

Fig. 1



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*Fig. 2*



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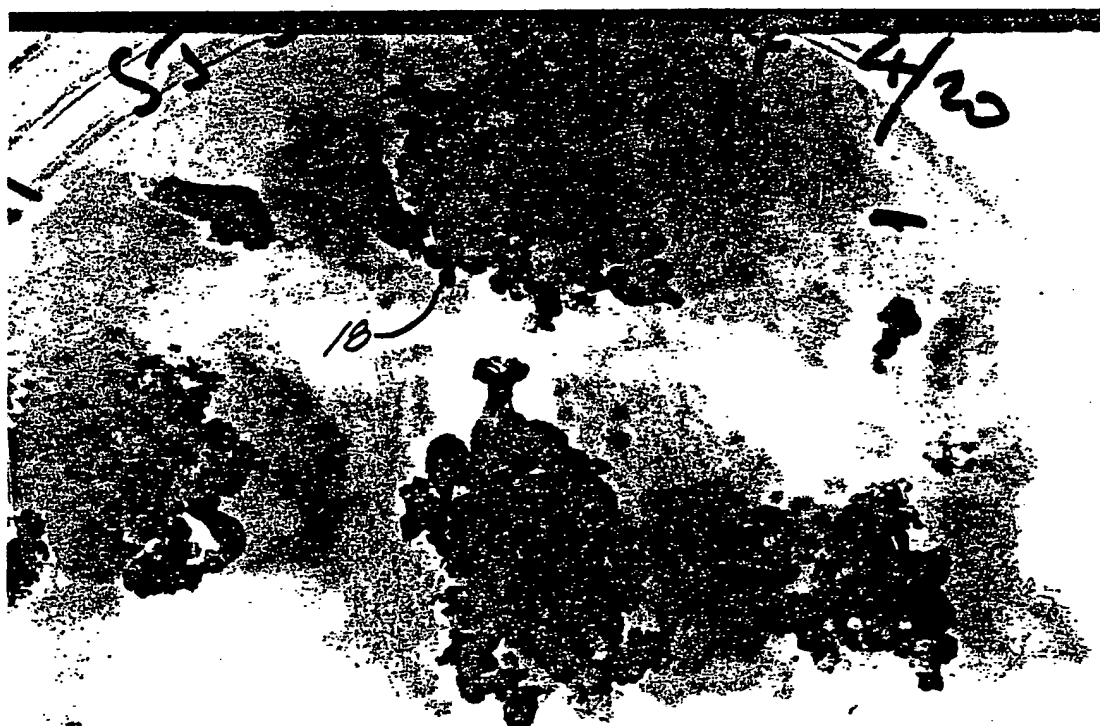
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*Fig. 3*



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*Fig. 4*

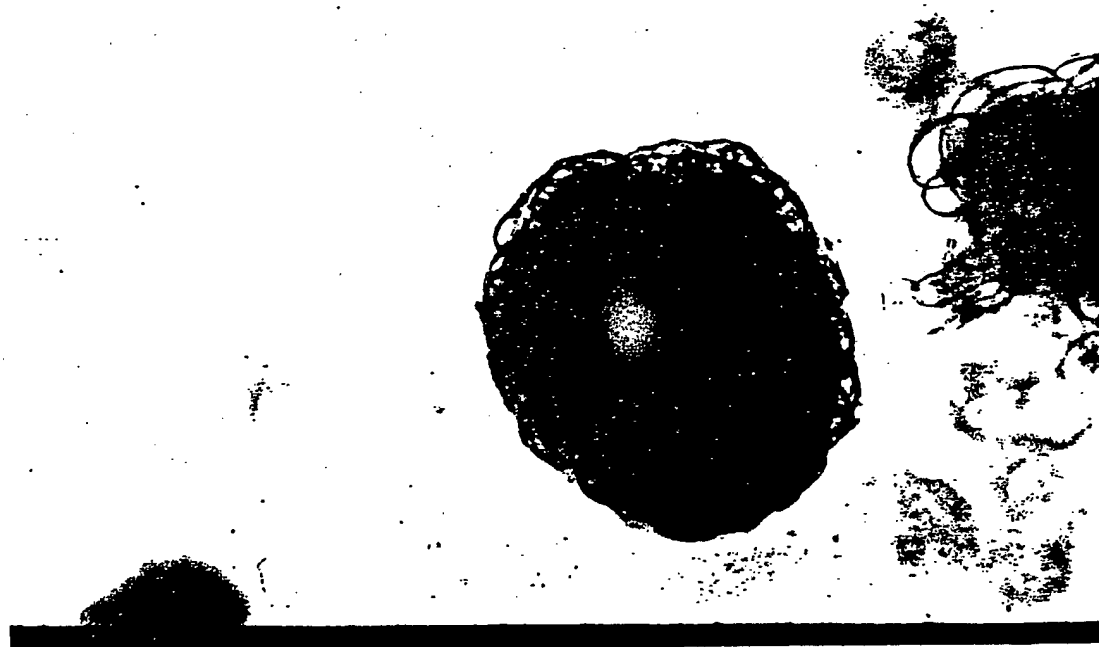


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*Fig. 5*

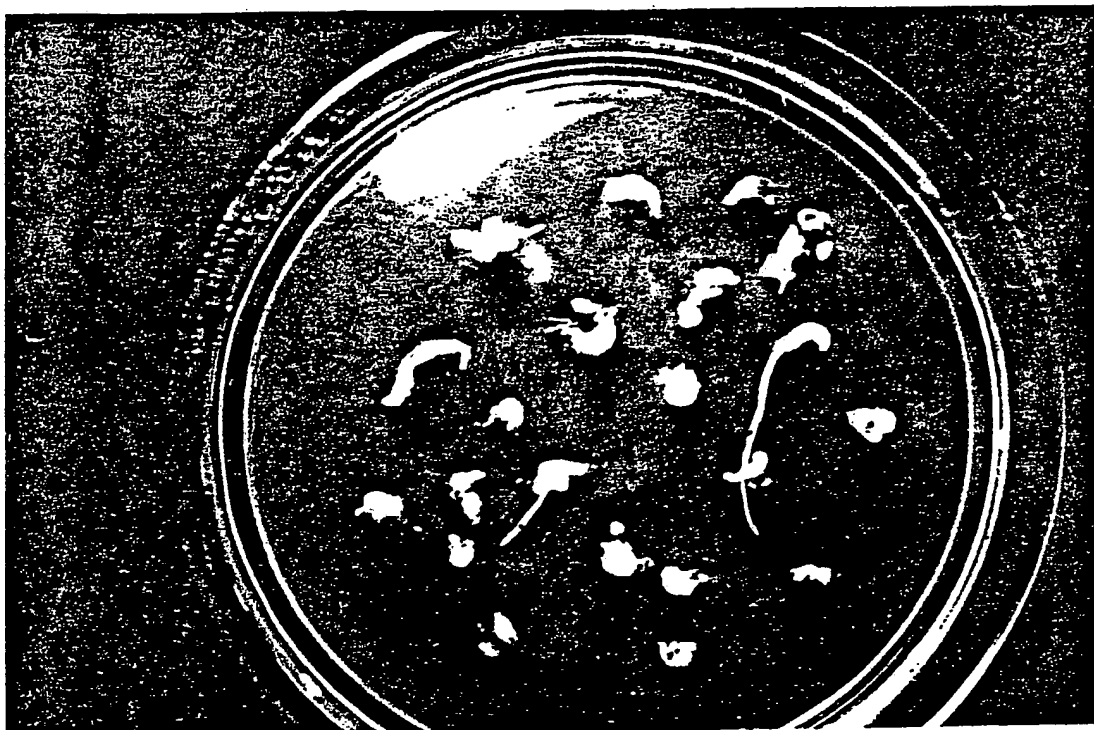


*Fig. 6*



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*Fig. 7*



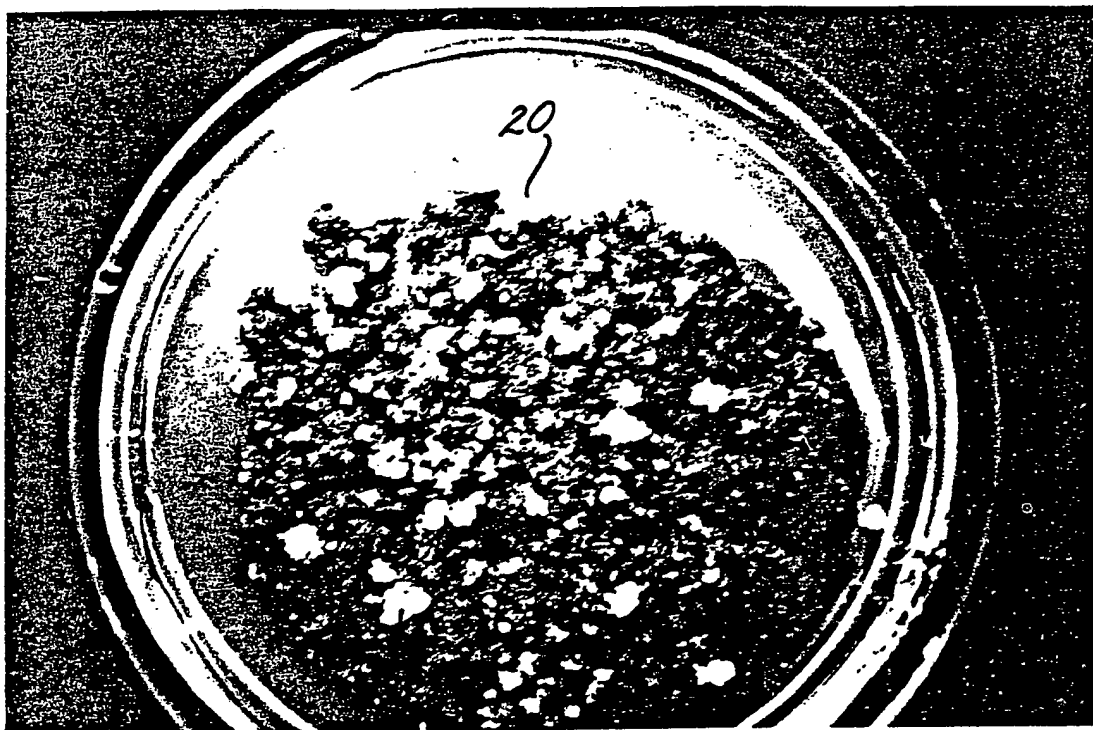
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16

*Fig. 8*



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*Fig. 9*

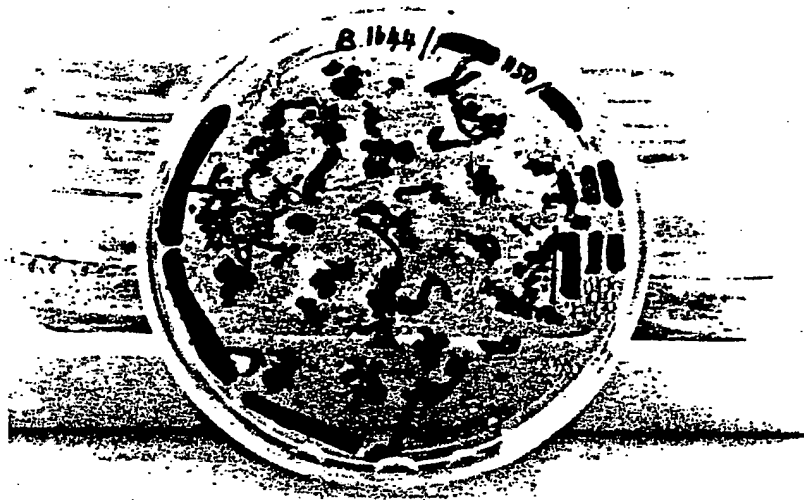


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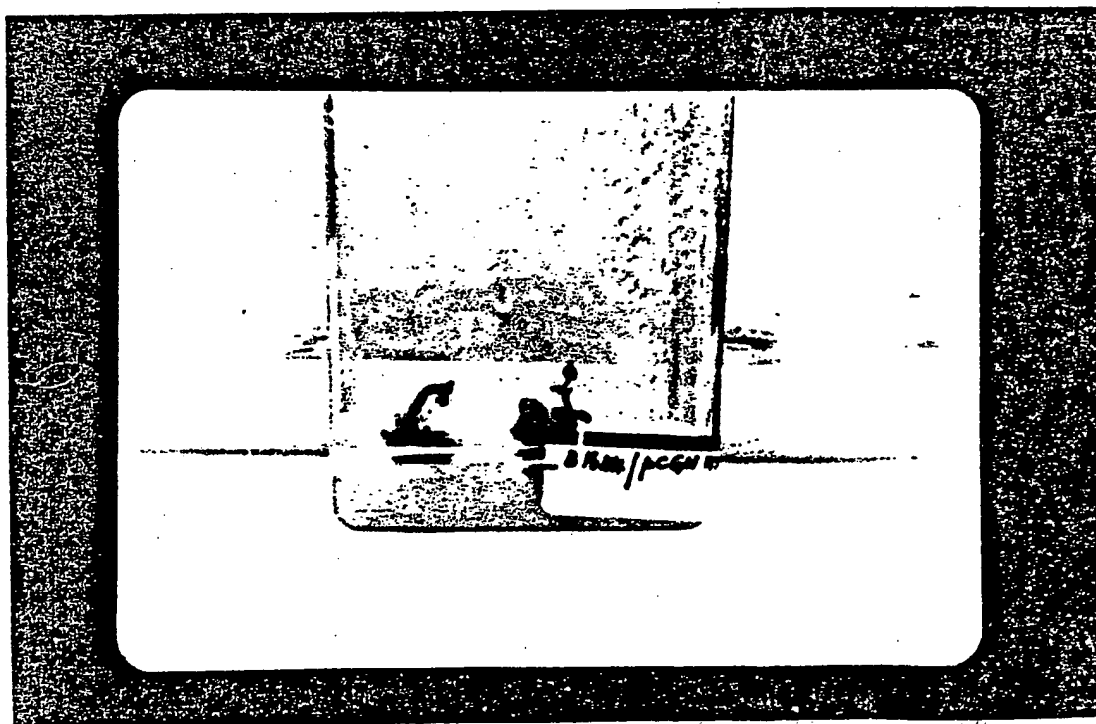
*Fig. 10*



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*Fig. 11*



*Fig. 12*





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*Fig. 13*



*Fig. 14*

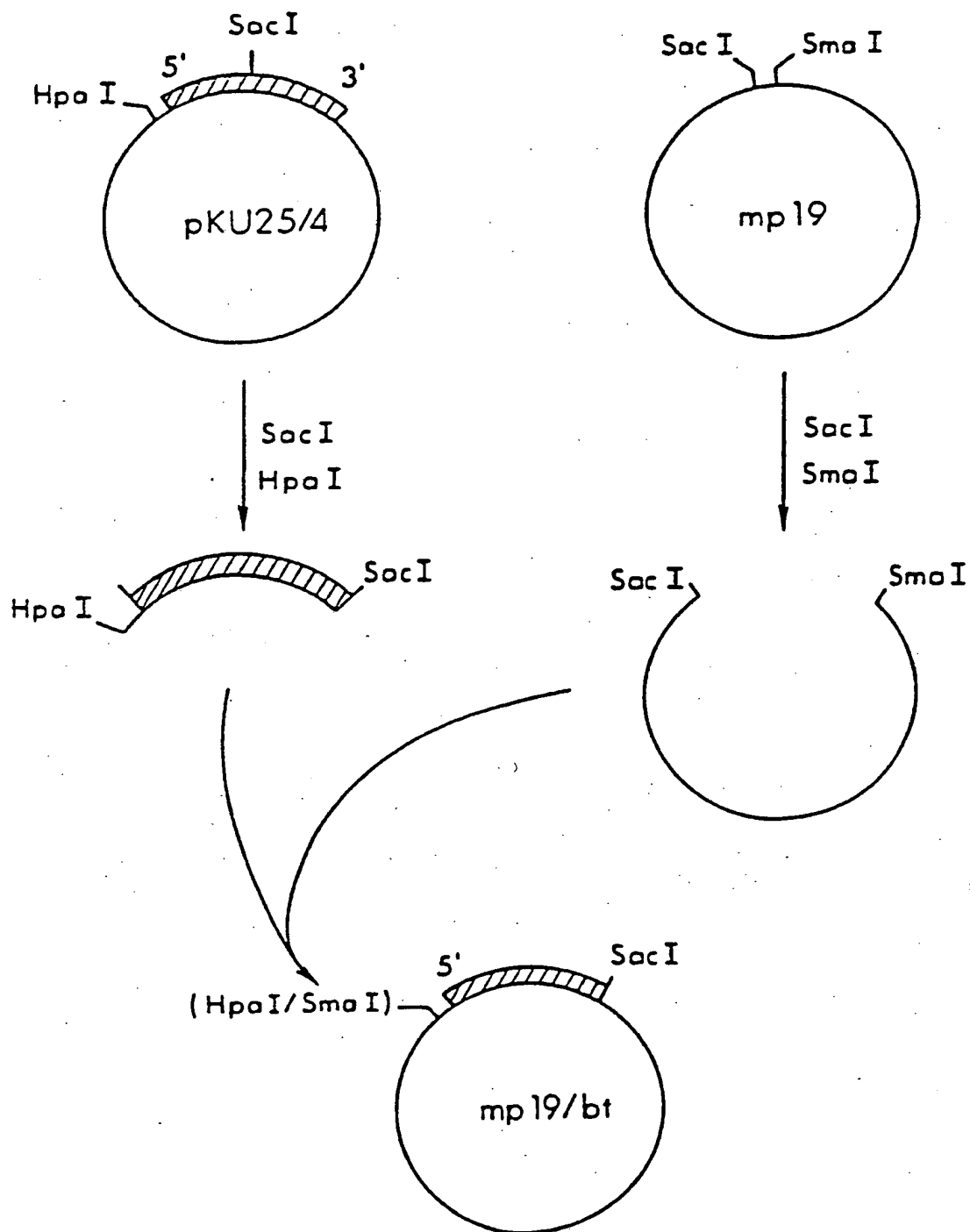


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*Fig. 15*

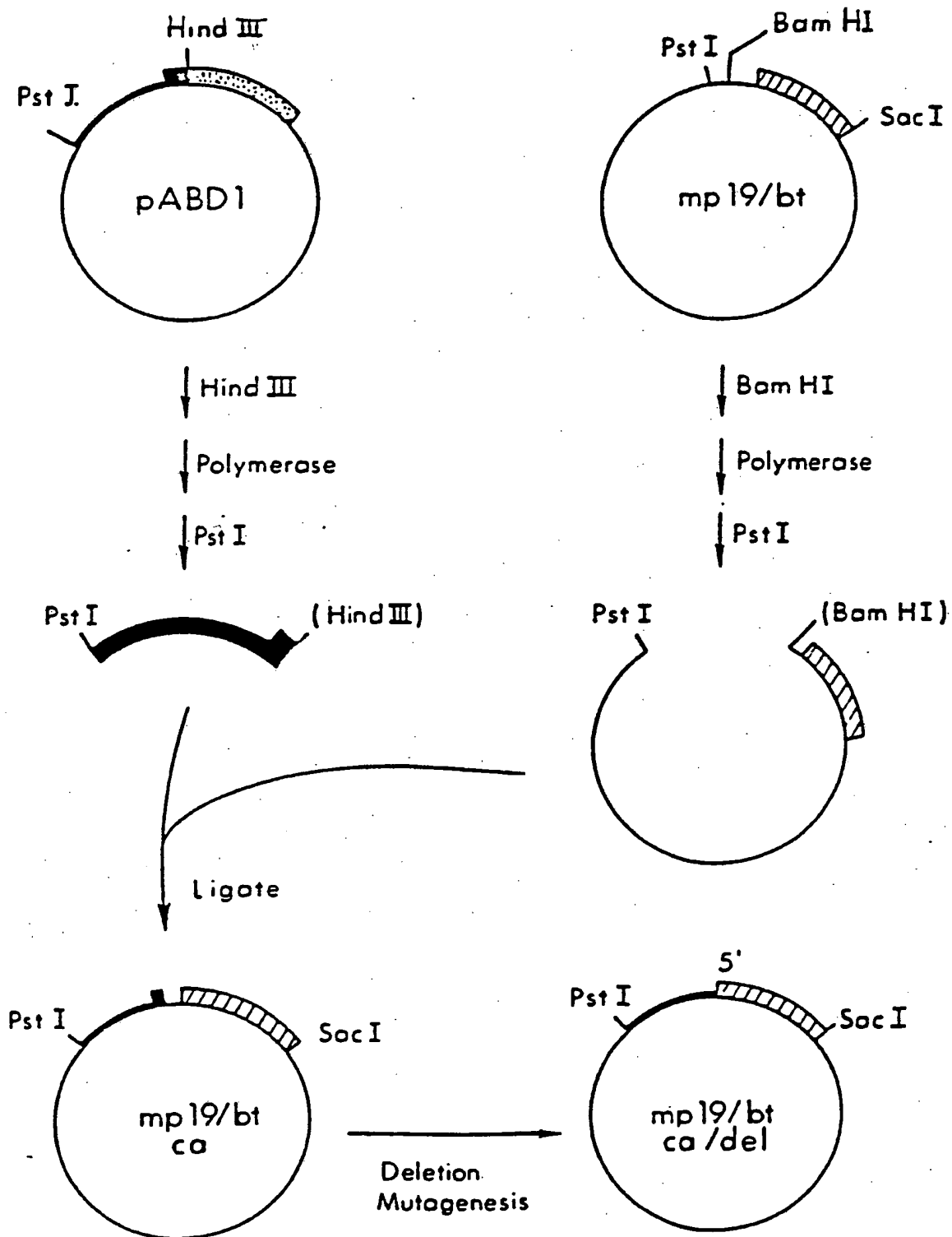


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*Fig. 16*

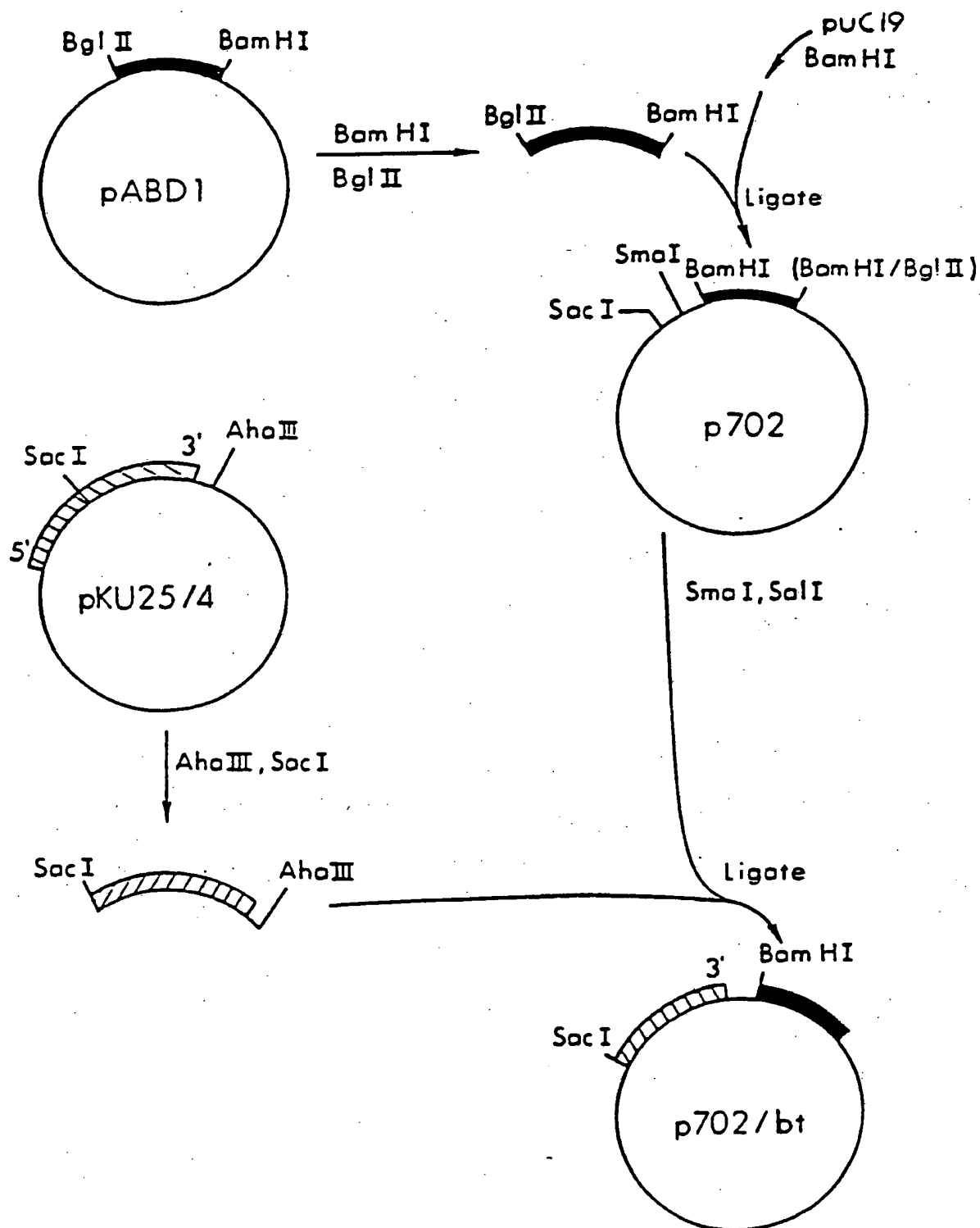
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Fig. 17



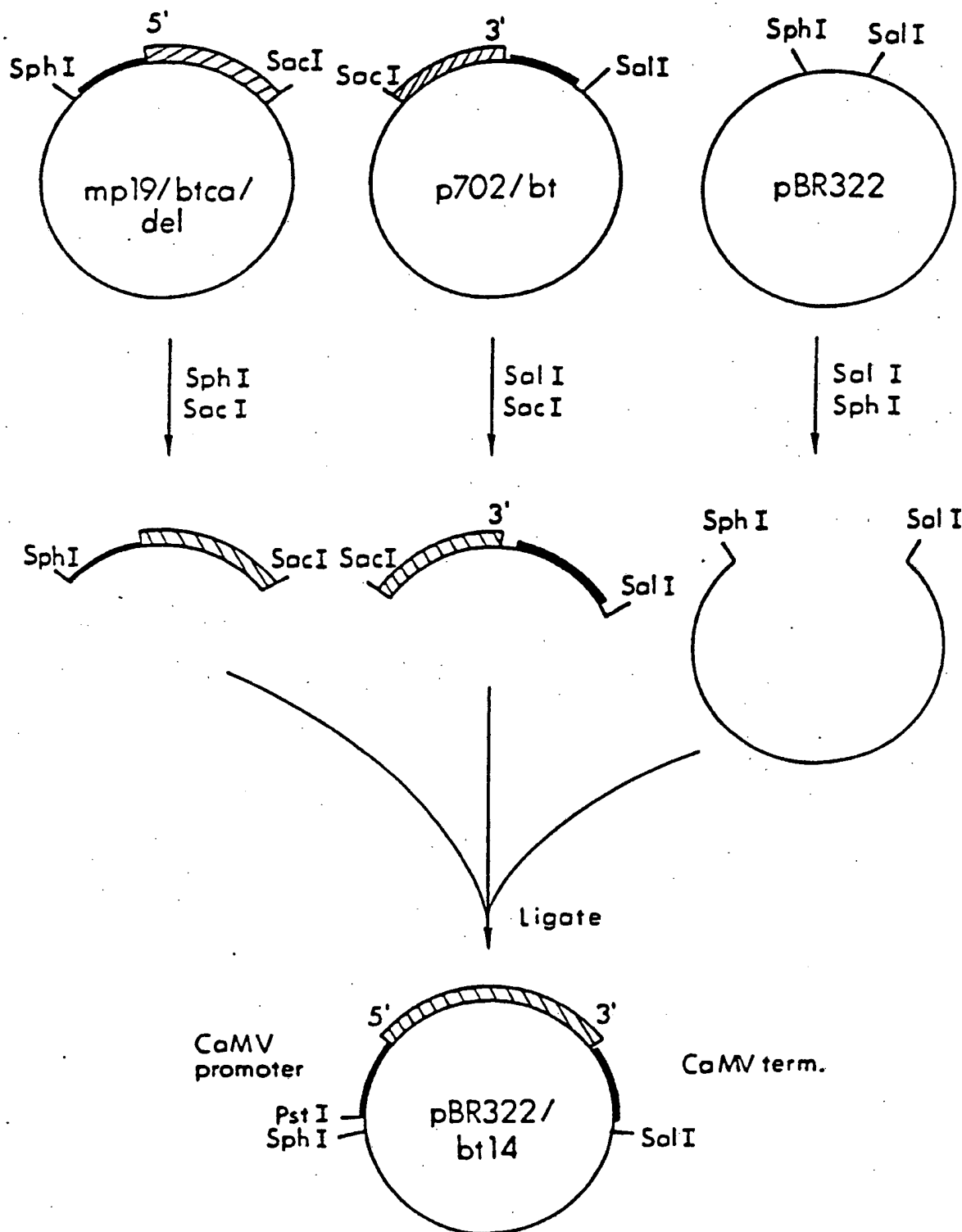
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Fig. 18.



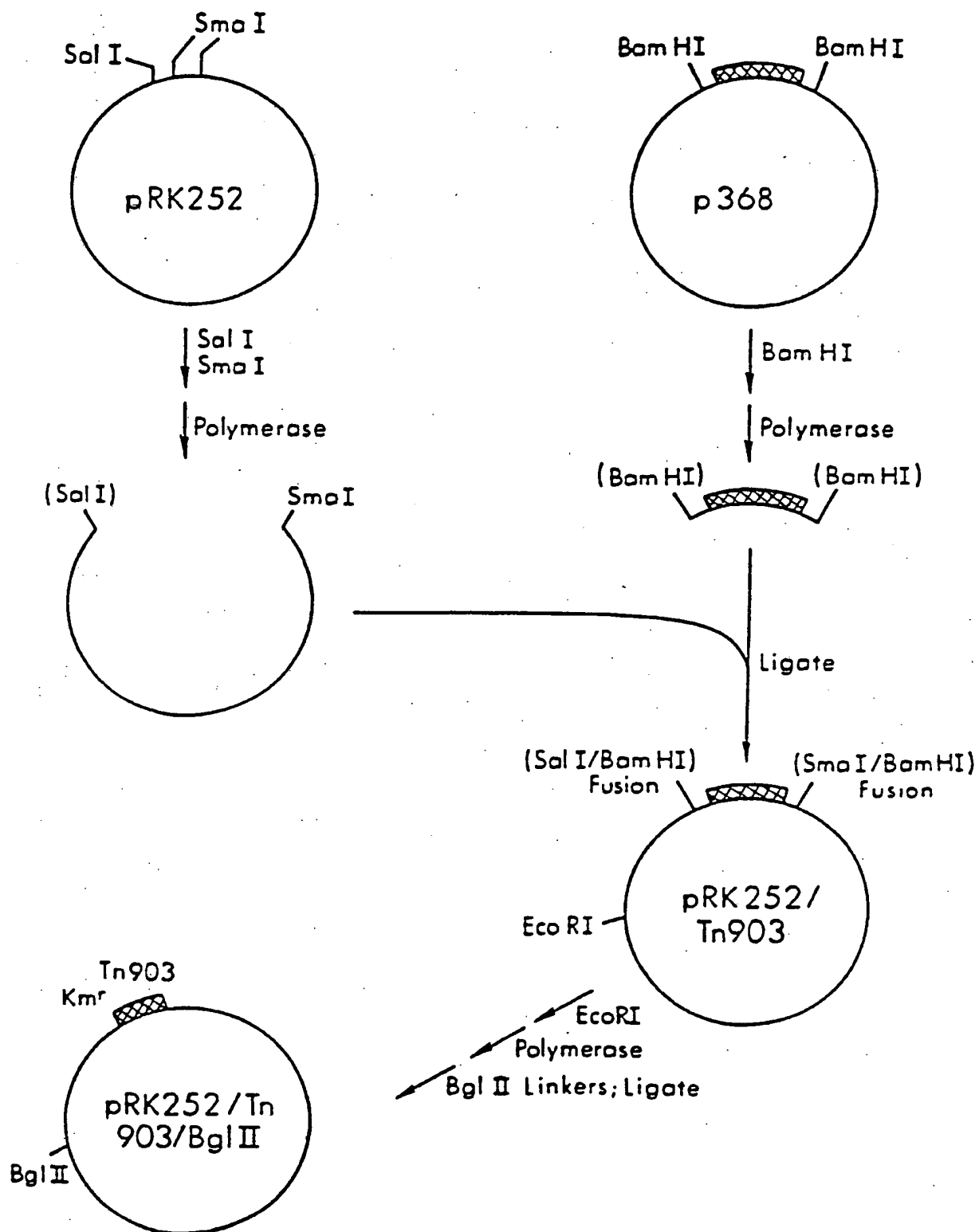
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Fig. 19



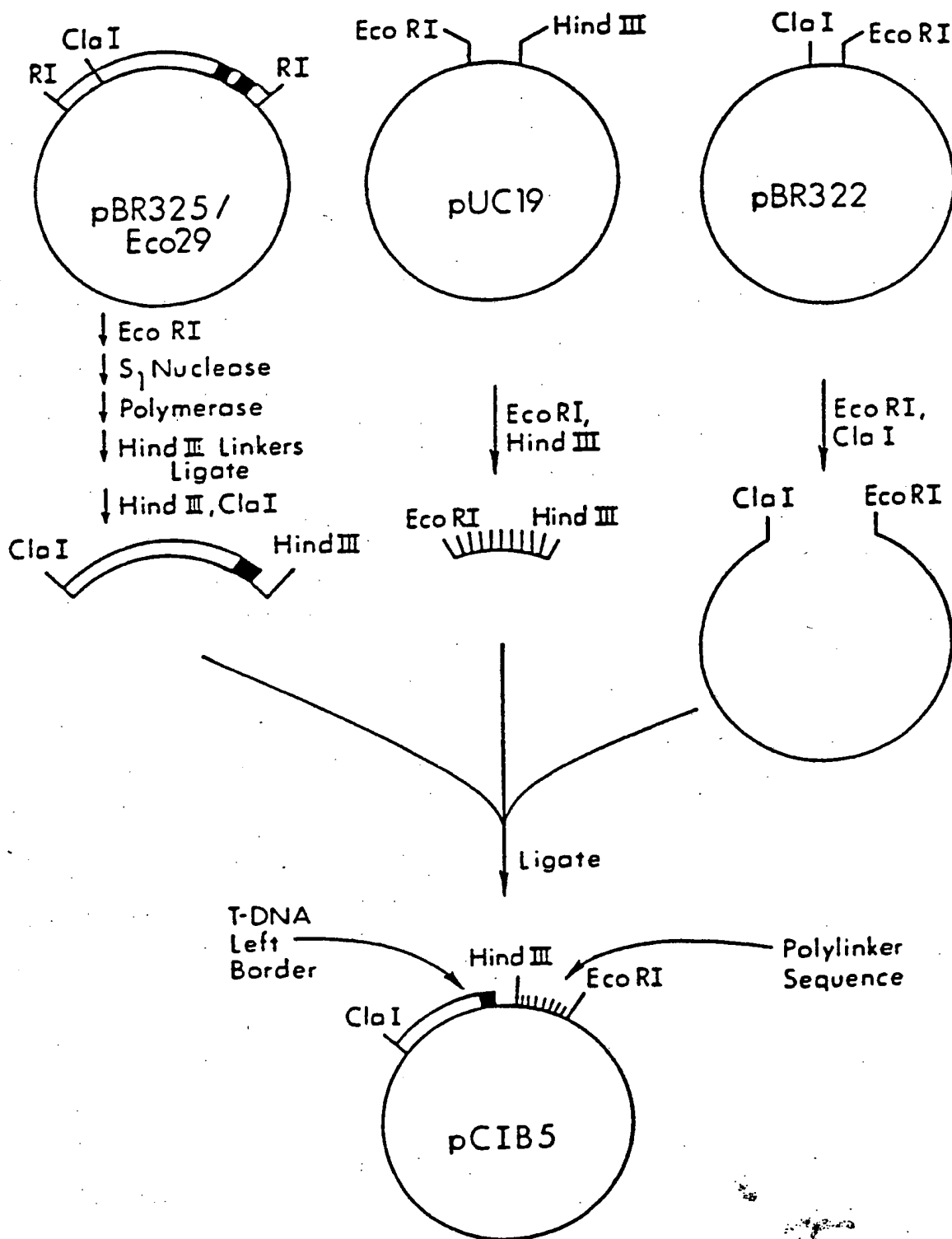
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Fig. 20



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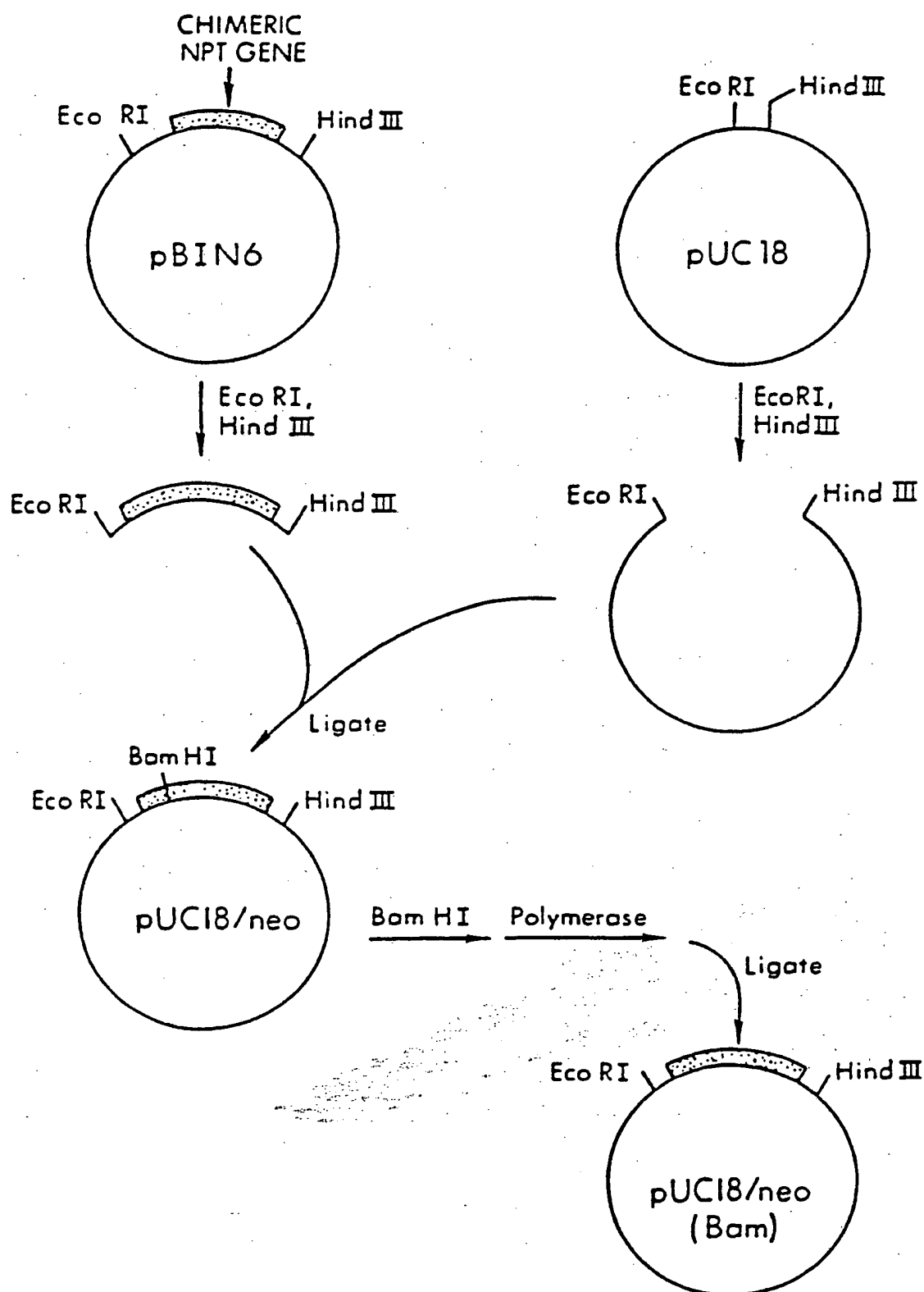
Fig. 21



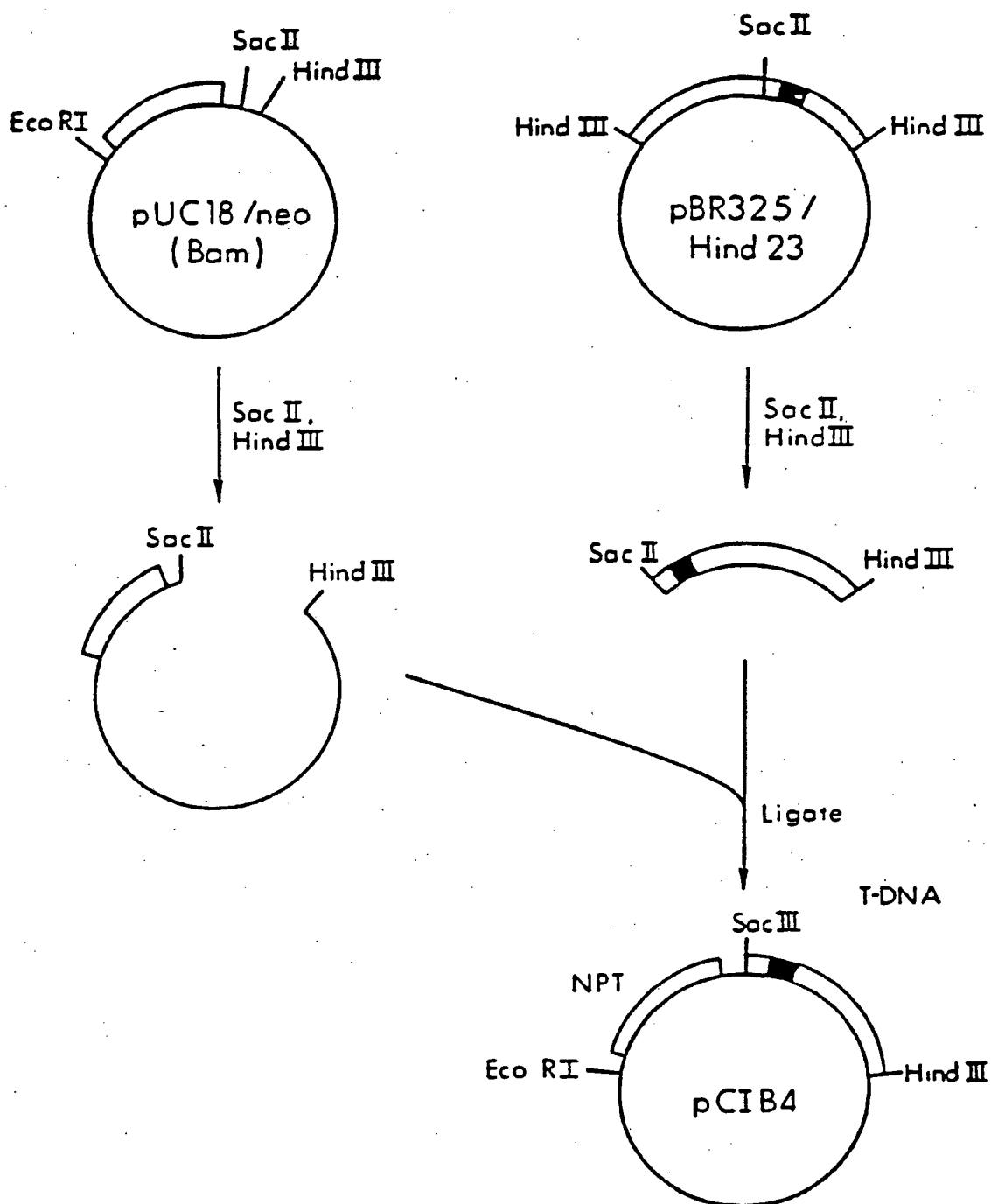


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Fig. 22

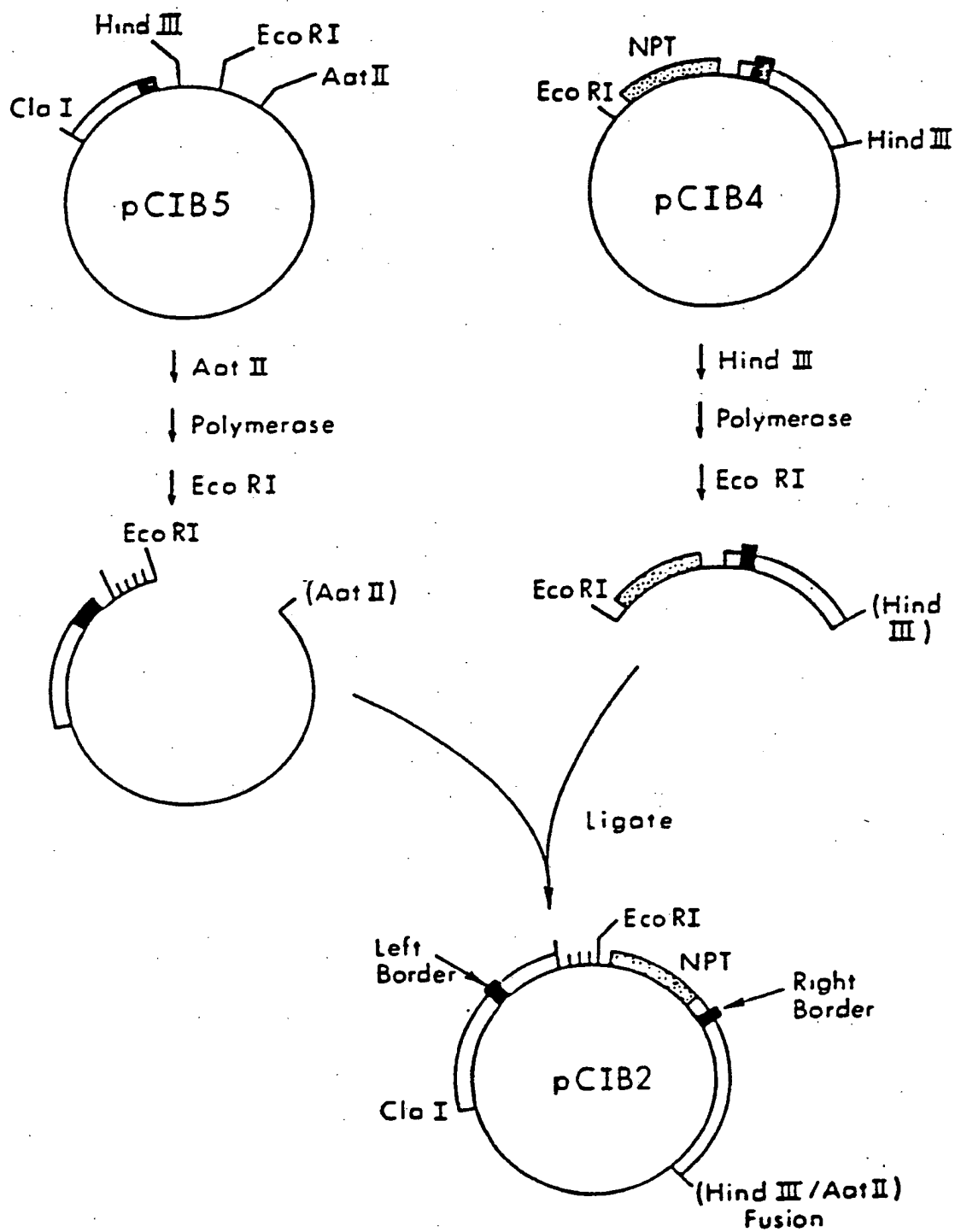


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*Fig. 23*

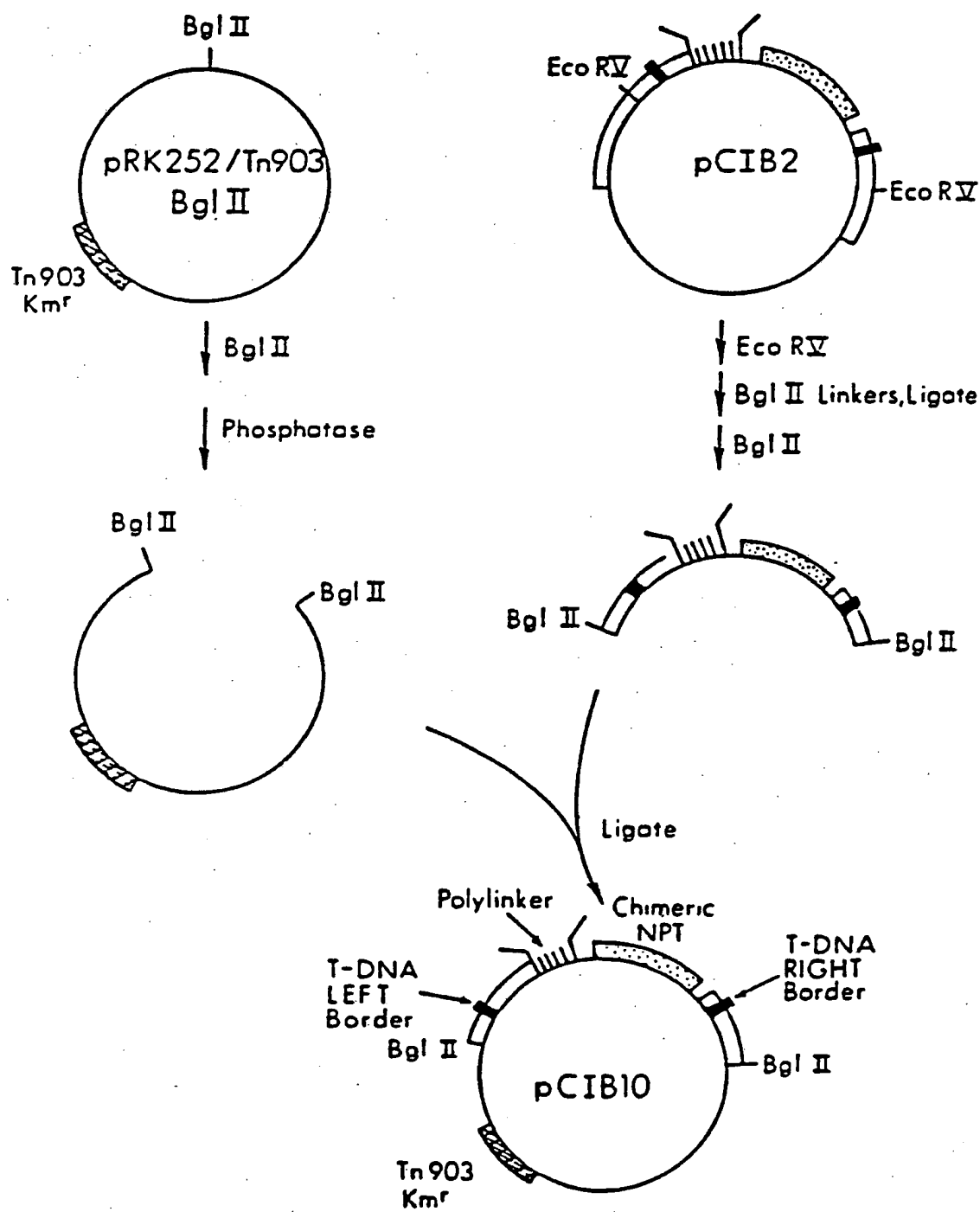
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Fig. 24



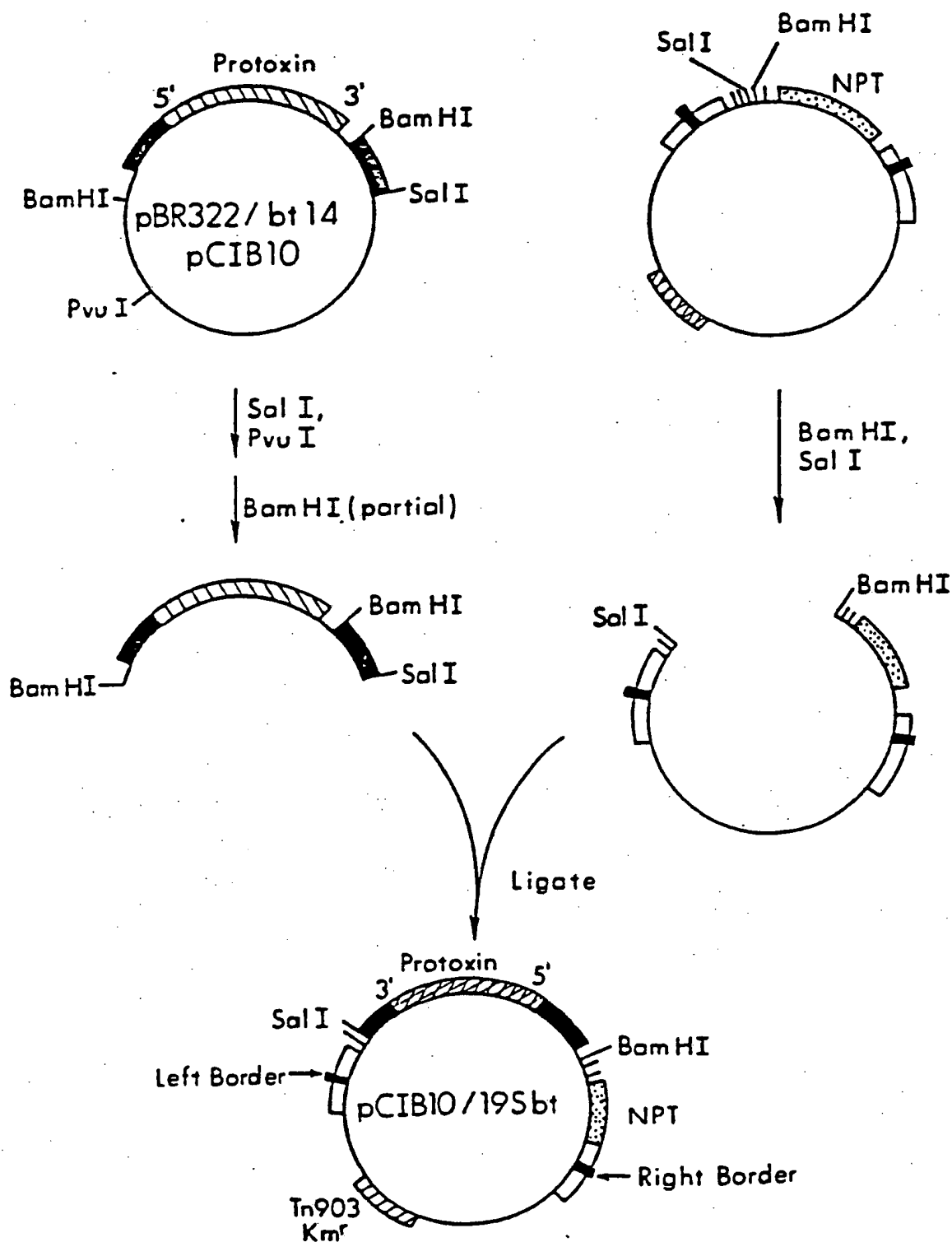
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Fig. 25

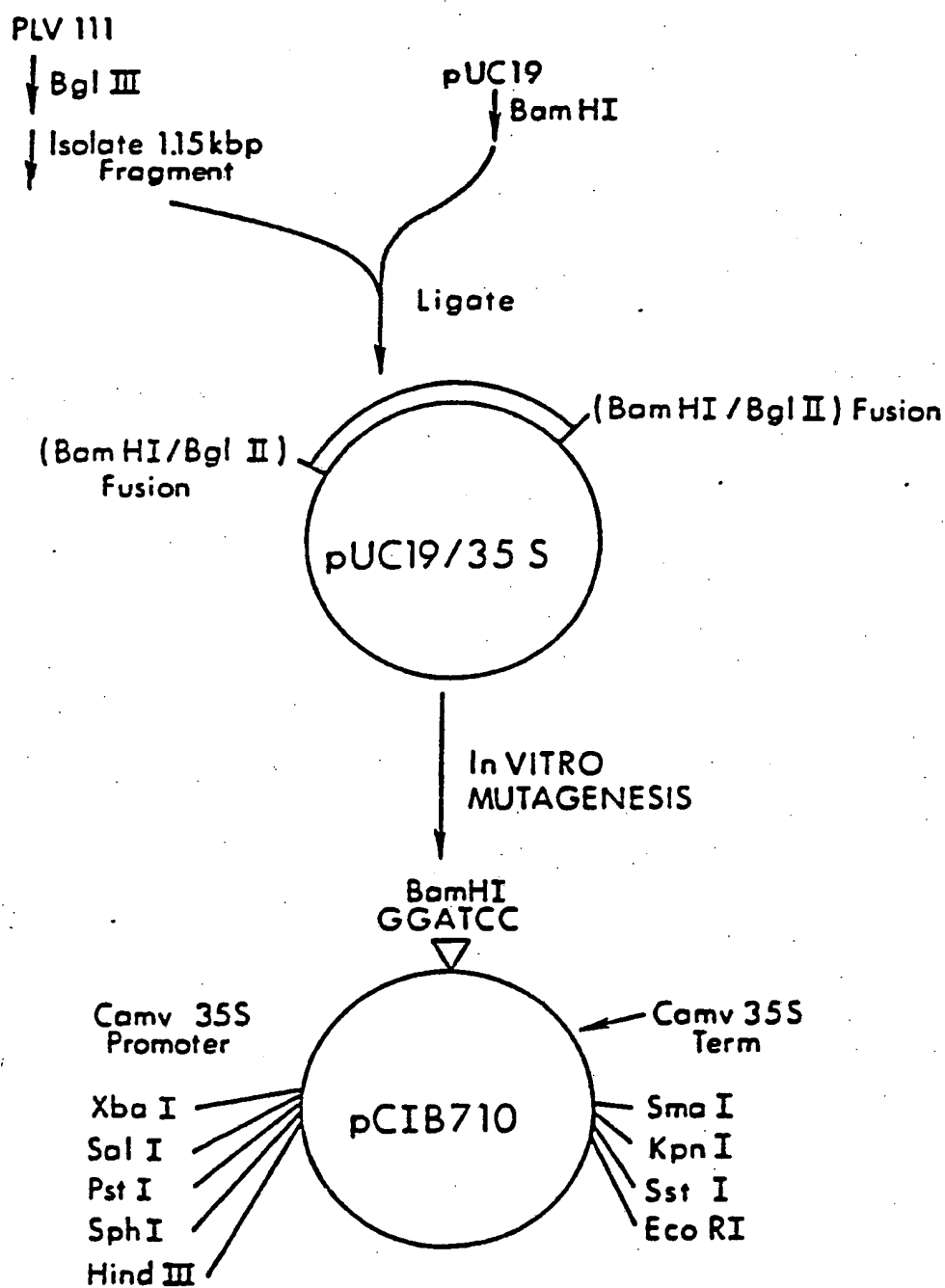


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Fig. 26

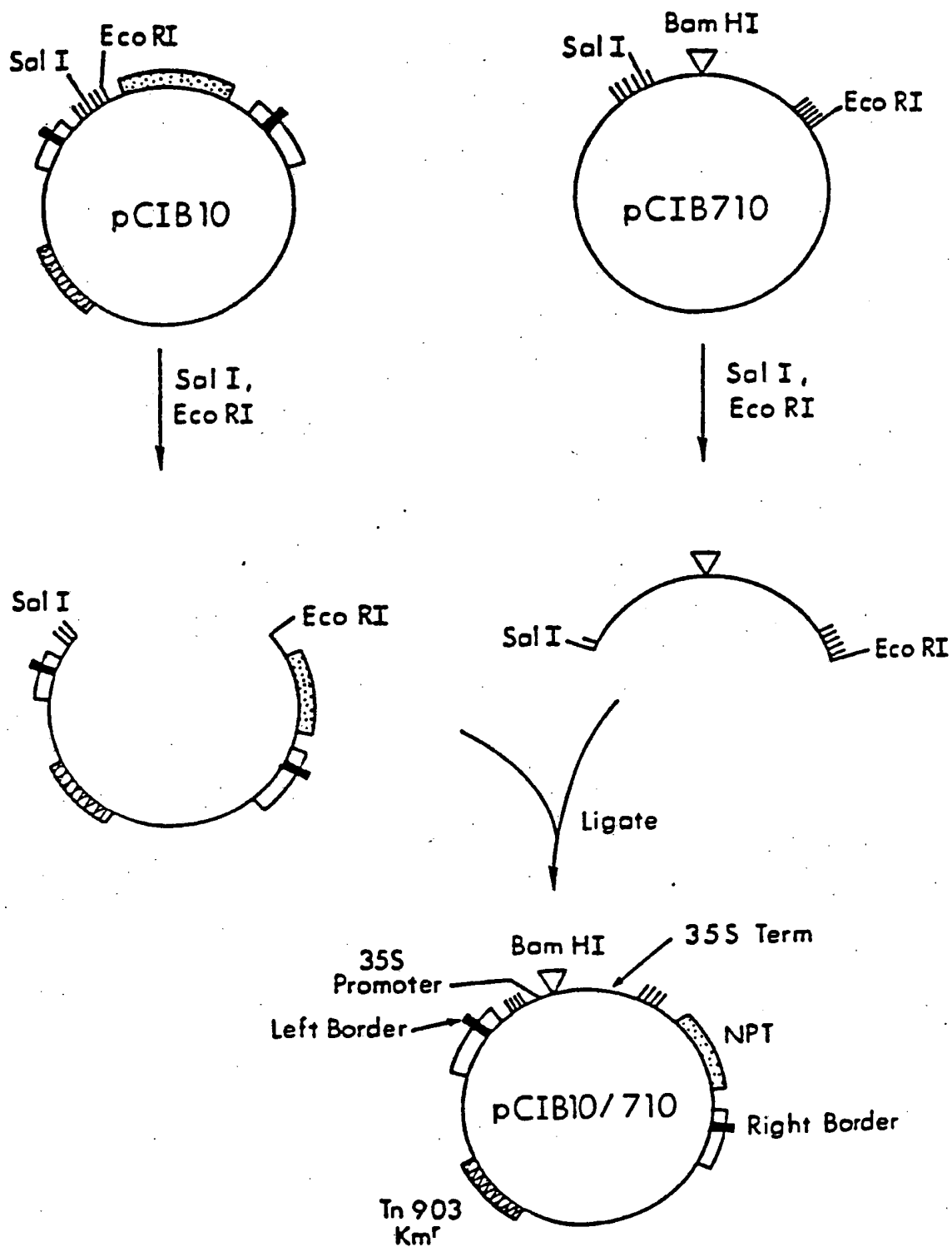


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*Fig. 27*

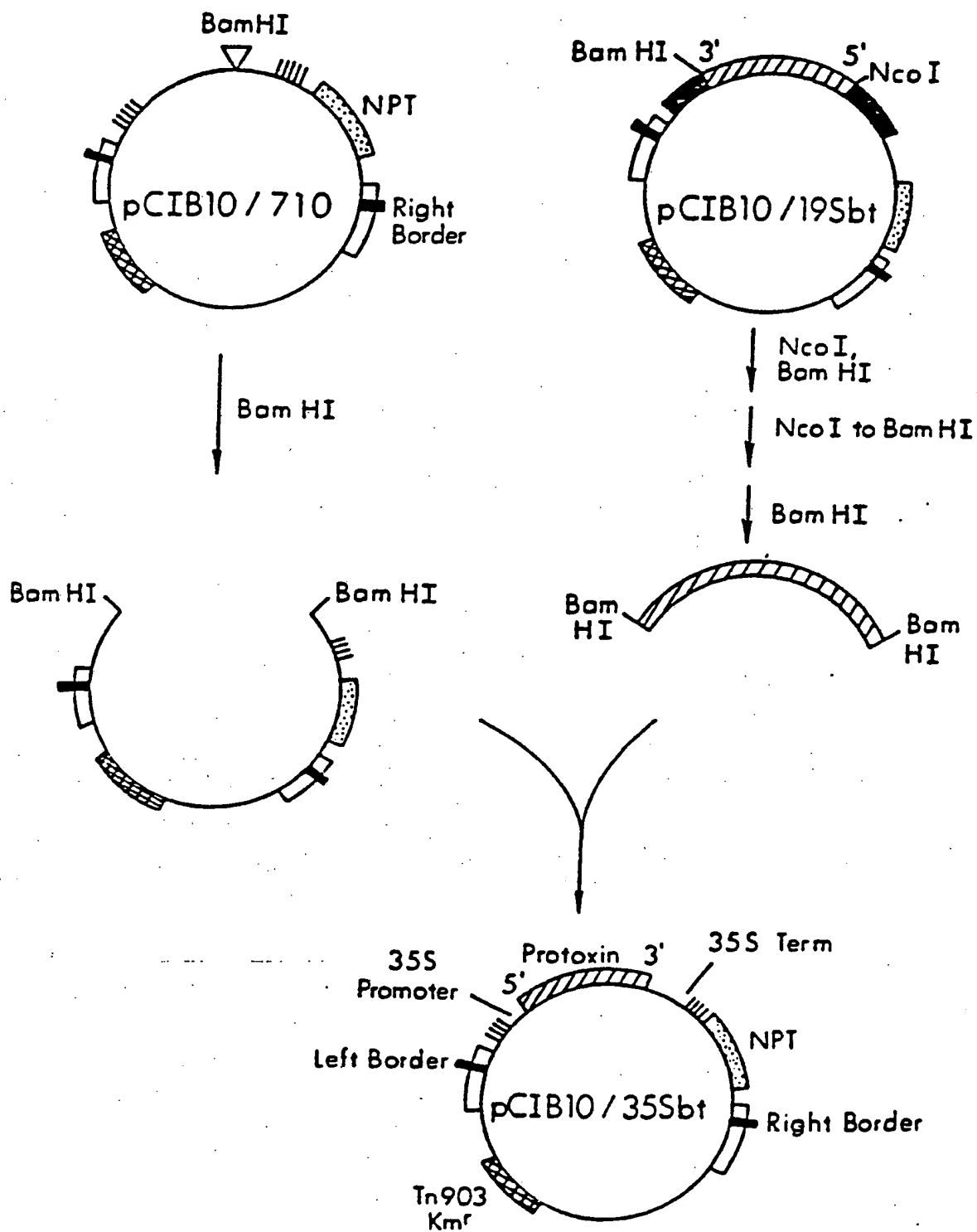
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Fig. 28



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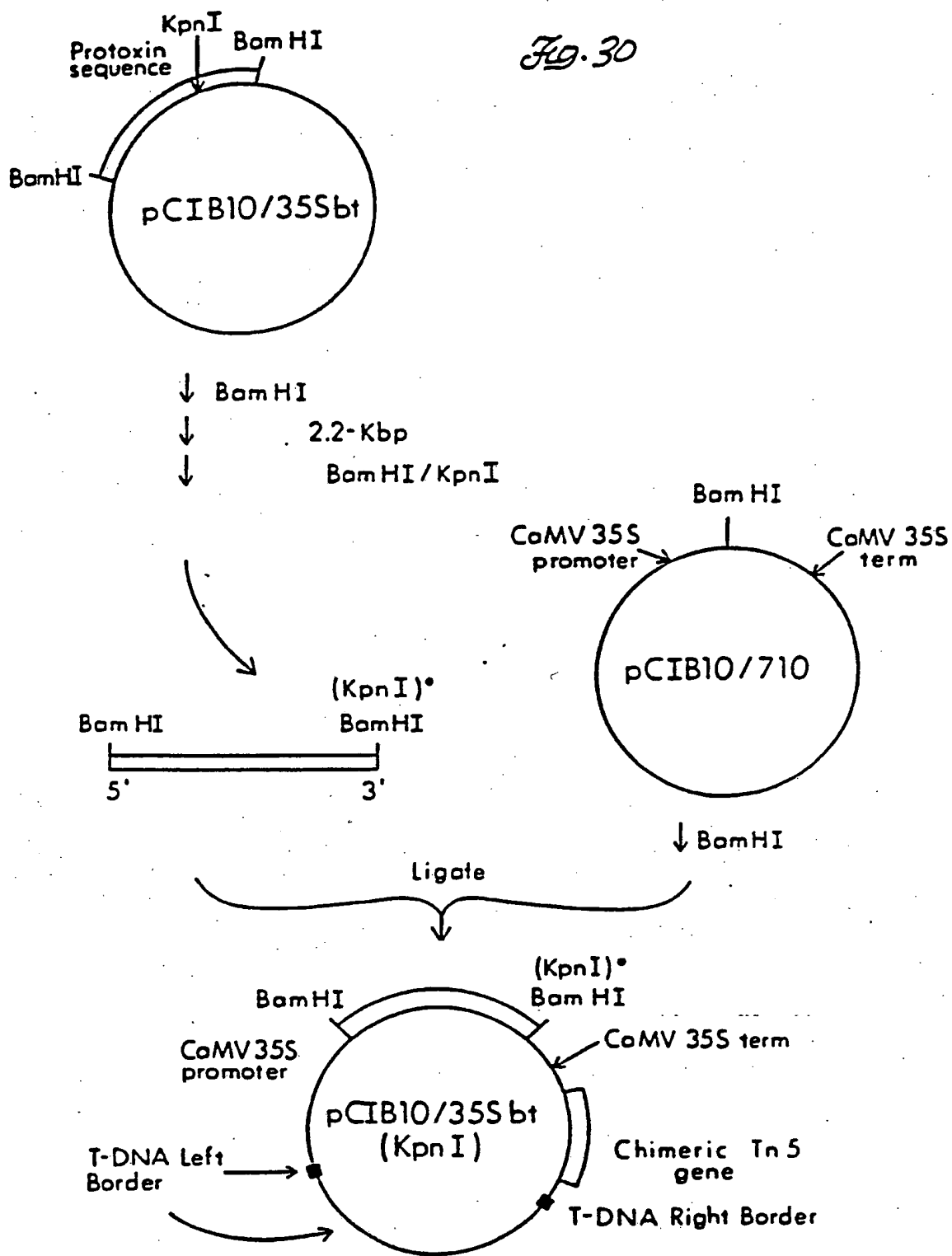
Fig. 29



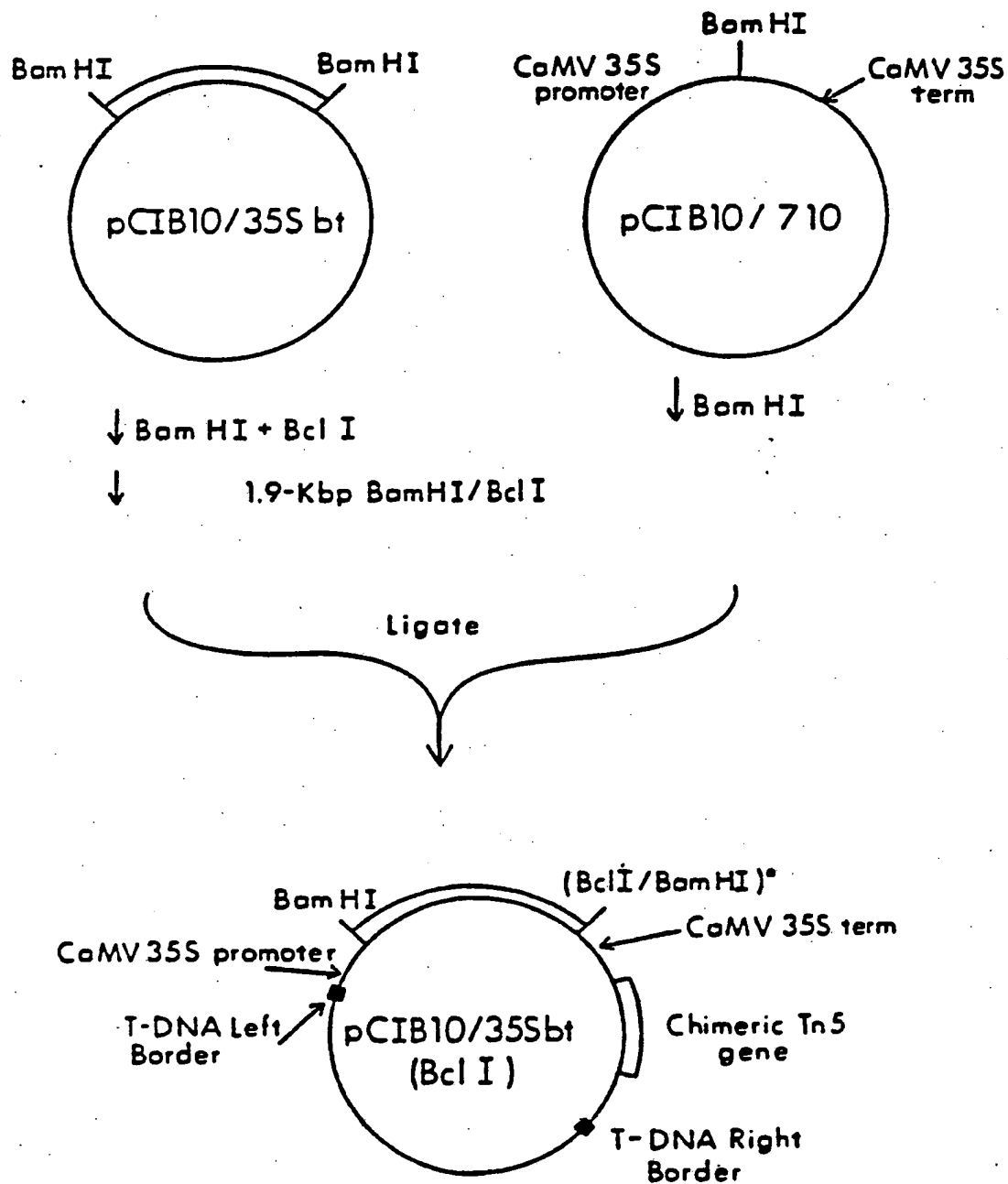


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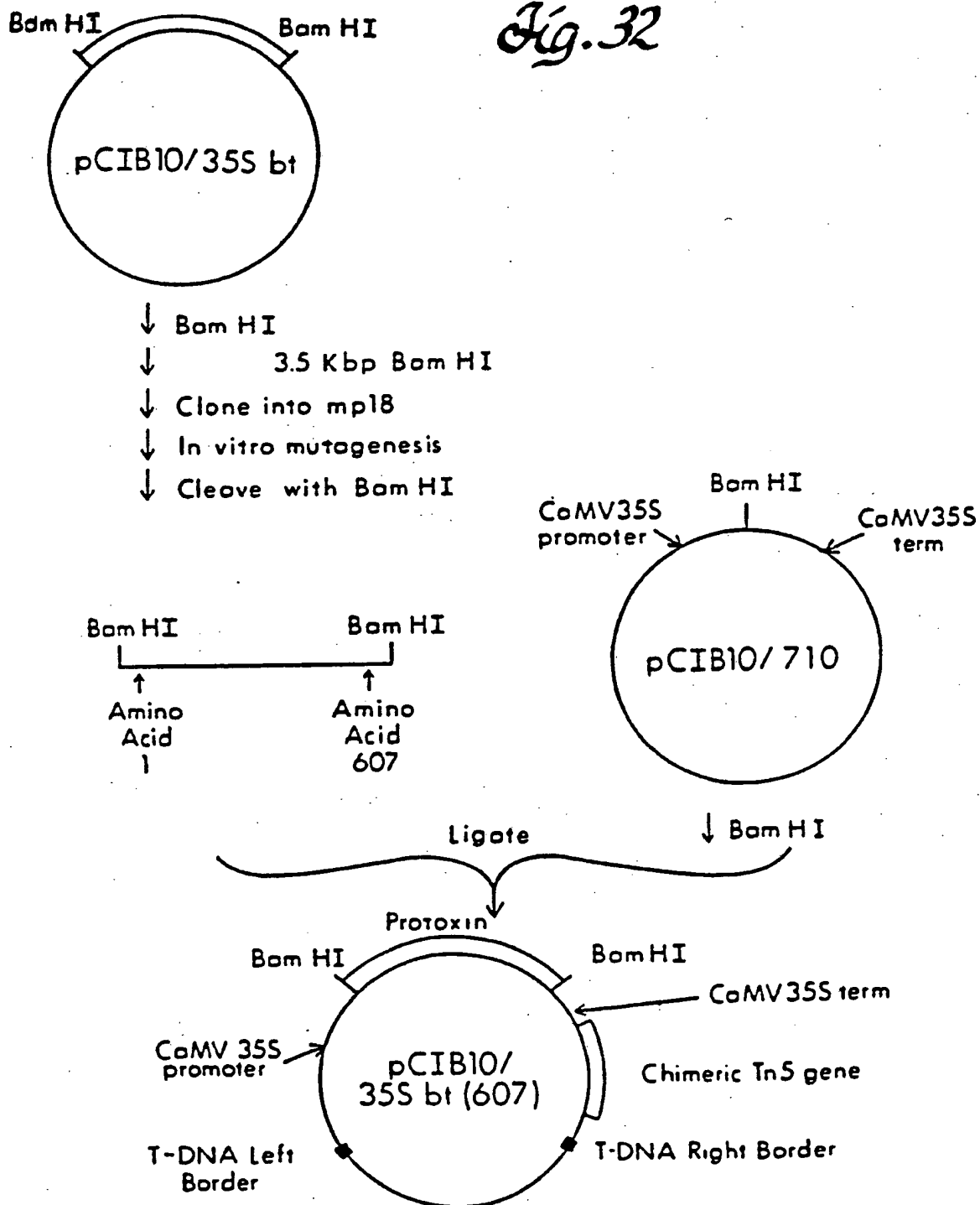
Fig. 30



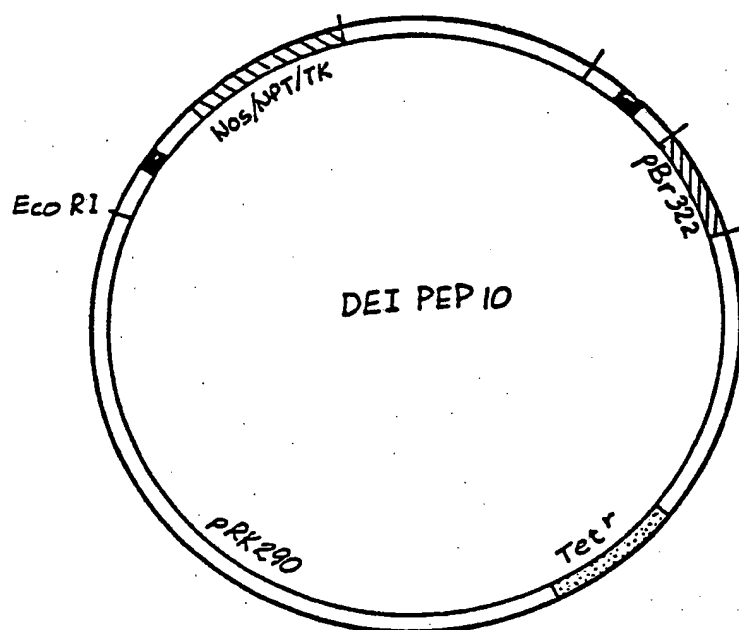
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*Fig. 31*

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*Fig. 32*

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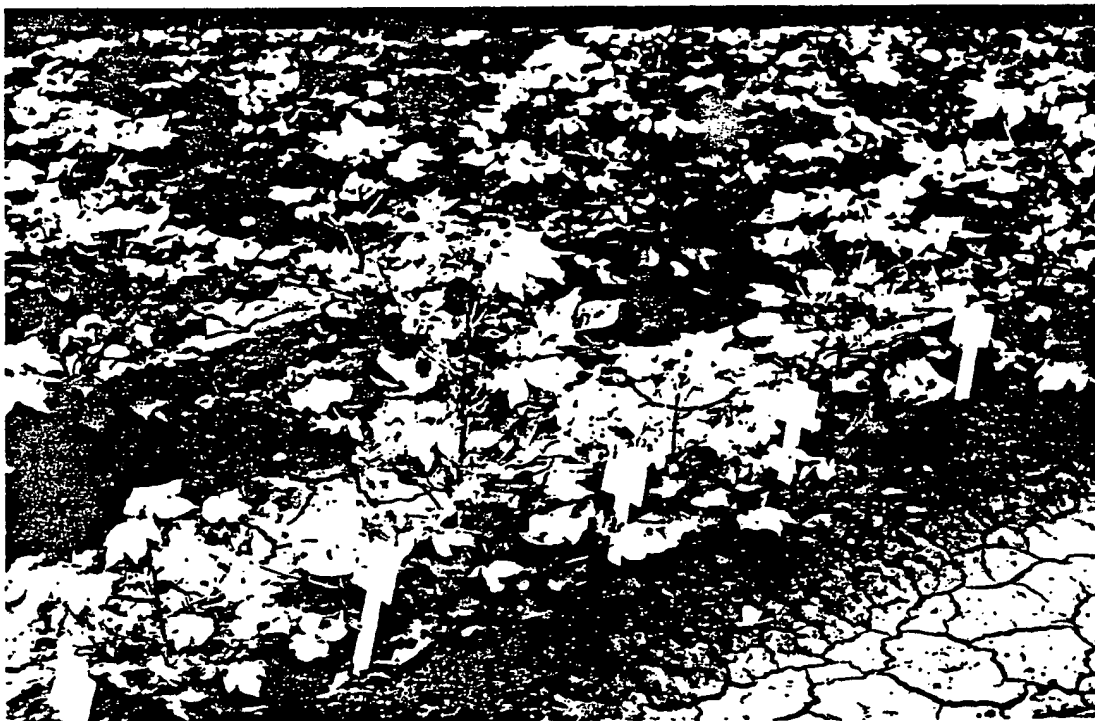
*Fig. 33*

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*Fig. 34*



*Fig. 35*



# INTERNATIONAL SEARCH REPORT

International Application No. **PCT/U88/04116**

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC <b>IPC(4): C12N 5/00; C12N 15/00; A01H 1/04;</b> <b>U.S. Cl.: 435/240.5; 435/172.3; 800/1; 435/320; 435/240.46</b>														
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched <sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%; border: 1px solid black; padding: 5px;">Classification System</th> <th style="border: 1px solid black; padding: 5px;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; padding: 5px; vertical-align: top;">U.S.</td> <td style="border: 1px solid black; padding: 5px;">435/240.5; 435/172.3; 800/1; 435/240.46; 435/320</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup></div> <p style="font-size: small;">Databases: Chemical Abstracts Services Online (File CA, 1969-1989). File Biosis, 1969-1989). Automated Patent System (File USPAT, 1975-1989). See attachment for search terms.</p>			Classification System	Classification Symbols	U.S.	435/240.5; 435/172.3; 800/1; 435/240.46; 435/320								
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<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border: 1px solid black; padding: 5px;">Category <sup>*</sup></th> <th style="width: 70%; border: 1px solid black; padding: 5px;">Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 20%; border: 1px solid black; padding: 5px;">Relevant to Claim No. <sup>13</sup></th> </tr> <tr> <td style="border: 1px solid black; padding: 5px; vertical-align: top;"> <div style="text-align: center;">X Y</div> </td> <td style="border: 1px solid black; padding: 5px; vertical-align: top;">           US, A, 4,672,035 (DAVIDONIS ET AL.)            09 June 1987. See the entire document.         </td> <td style="border: 1px solid black; padding: 5px; vertical-align: top; text-align: center;"> <div style="text-align: center;">1,4 2,3, 5-78</div> </td> </tr> <tr> <td style="border: 1px solid black; padding: 5px; vertical-align: top;"> <div style="text-align: center;">Y</div> </td> <td style="border: 1px solid black; padding: 5px; vertical-align: top;">           Chemical Abstracts, Volume 100, No. 9,            issued 1984, February 27 (Columbus,            Ohio, USA), Davidonis et al., "Plant            regeneration from callus tissue of            Gossypium hirsutum L.," see page 343,            column 2, the abstract no. 65175p,            Sci. Lett. 1983, 32(1-2): 89-93 (Eng.).         </td> <td style="border: 1px solid black; padding: 5px; vertical-align: top; text-align: center;"> <div style="text-align: center;">1-78</div> </td> </tr> <tr> <td style="border: 1px solid black; padding: 5px; vertical-align: top;"> <div style="text-align: center;">Y</div> </td> <td style="border: 1px solid black; padding: 5px; vertical-align: top;">           Biological Abstracts, Volume 78, No. 8,            issued 15 October 1984 (Philadelphia,            Pennsylvania, USA), Stuart et al.,            "Somatic embryogenesis from cell cultures            of Medicago sativa: 2: The interaction of            amino acids with ammonium," see page            6339, column 1, the abstract no. 56366,            Plant Sci. Lett. 1984, 34(1/2): 175-182            (Eng.).         </td> <td style="border: 1px solid black; padding: 5px; vertical-align: top; text-align: center;"> <div style="text-align: center;">1-78</div> </td> </tr> </table>			Category <sup>*</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	<div style="text-align: center;">X Y</div>	US, A, 4,672,035 (DAVIDONIS ET AL.) 09 June 1987. See the entire document.	<div style="text-align: center;">1,4 2,3, 5-78</div>	<div style="text-align: center;">Y</div>	Chemical Abstracts, Volume 100, No. 9, issued 1984, February 27 (Columbus, Ohio, USA), Davidonis et al., "Plant regeneration from callus tissue of Gossypium hirsutum L.," see page 343, column 2, the abstract no. 65175p, Sci. Lett. 1983, 32(1-2): 89-93 (Eng.).	<div style="text-align: center;">1-78</div>	<div style="text-align: center;">Y</div>	Biological Abstracts, Volume 78, No. 8, issued 15 October 1984 (Philadelphia, Pennsylvania, USA), Stuart et al., "Somatic embryogenesis from cell cultures of Medicago sativa: 2: The interaction of amino acids with ammonium," see page 6339, column 1, the abstract no. 56366, Plant Sci. Lett. 1984, 34(1/2): 175-182 (Eng.).	<div style="text-align: center;">1-78</div>
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<div style="display: flex; justify-content: space-between; font-size: x-small;"> <div style="width: 45%;"> <p><sup>*</sup> Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 50%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>														
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border: 1px solid black; padding: 5px;">           Date of the Actual Completion of the International Search   <div style="text-align: center; font-size: large;">24 March 1989</div> </td> <td style="width: 50%; border: 1px solid black; padding: 5px;">           Date of Mailing of this International Search Report   <div style="text-align: center; font-size: large;">04 MAY 1989</div> </td> </tr> <tr> <td style="border: 1px solid black; padding: 5px;">           International Searching Authority   <div style="text-align: center; font-size: large;">ISA/US</div> </td> <td style="border: 1px solid black; padding: 5px;">           Signature of Authorized Officer  <div style="text-align: center;">               Charles E. Cohen           </div> </td> </tr> </table>			Date of the Actual Completion of the International Search  <div style="text-align: center; font-size: large;">24 March 1989</div>	Date of Mailing of this International Search Report  <div style="text-align: center; font-size: large;">04 MAY 1989</div>	International Searching Authority  <div style="text-align: center; font-size: large;">ISA/US</div>	Signature of Authorized Officer <div style="text-align: center;">               Charles E. Cohen           </div>								
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**FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET**

**V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>1</sup>**

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers \_\_\_\_\_, because they relate to subject matter <sup>12</sup> not required to be searched by this Authority, namely:
  
2. ☐ Claim numbers \_\_\_\_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out <sup>13</sup>, specifically:
  
3. ☐ Claim numbers \_\_\_\_\_, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

**VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>2</sup>**

This International Searching Authority found multiple inventions in this international application as follows:

**See Attachment**

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
  
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
  
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

**Remark on Protest**

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Trolinder et al., <u>Proceedings of the Beltwide Cotton Production Research Conferences</u> , January 6-11, 1985, New Orleans, LA., page 46.	1-78
Y	Biological Abstracts, Volume 82, No. 9, issued 01 November 1986, (Philadelphia, Pennsylvania, USA), Shoemaker et al., "Characterization of somatic embryogenesis and plant regeneration in cotton ( <i>Gossypium hirsutum</i> L.)," see page AB-907, Column 2, the abstract no. 86768, Plant Cell Rep. 1986, 5(3): 178-181 (Eng.).	1-78
Y	Chemical Abstracts, Volume 106, No. 9, issued 1987, March 02 (Columbus, Ohio, USA), N.L.G. Trolinder, "Somatic embryogenesis and plant regeneration in <i>Gossypium hirsutum</i> L.," see page 225, column 1, the abstract no. 62935z, Diss. Abstr. Int. B 1986, 47(6): 2250-1 (Eng.).	1-78
Y	Chemical Abstracts, Volume 107, No. 5, issued 1987, August 03 (Columbus, Ohio, USA), Trolinder et al., "Somatic embryogenesis and plant regeneration in cotton ( <i>Gossypium hirsutum</i> L.)," see page 369, column 1, the abstract no. 36159a, Plant Cell Rep. 1987, 6(3): 231-4 (Eng.).	1-78
Y	Chemical Abstracts, Volume 106, No. 21, issued 1987, May 25 (Columbus, Ohio, USA), Umbeck et al., "Genetically transformed cotton ( <i>Gossypium hirsutum</i> L.) plants," see page 179, column 1, the abstract no. 170182k, Bio/Technology 1987, 5(3): 263-6 (Eng.).	58-79, 85-86
Y	Chemical Abstracts, Volume 108, No. 11, issued 1988, March 14 (Columbus, Ohio, USA), Firoozabady et al., "Transformation of cotton ( <i>Gossypium hirsutum</i> L.) by <i>Agrobacterium tumefaciens</i> and regeneration of transgenic plants," see page 153, column 1, the abstract no. 88857s, Plant Mol. Biol. 1987, 10(2): 105-16 (Eng.).	58-79, 85-86



## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	The EMBO Journal, Volume 3, No. 8, issued August 1984, (Oxford, England), De Block et al., "Expression of foreign genes in regenerated plants and in their progeny" pages 1681-1689. See entire document	58-79, 85-86
Y	Chemical Abstracts, Volume 98, No. 23, issued 1983, June 6 (Columbus, Ohio, USA), Becker et al., "Herpes simplex virus type 1 thymidine kinase gene expression in Escherichia coli, "see page 168, column 2, the abstract no. 192666q, Gene 1983, 21(1-2): 51-8 (Eng.).	86
Y	Chemical Abstracts, Volume 100, No. 9, issued 1984, February 27 (Columbus, Ohio, USA), Kit et al., "Nucleotide sequence of the herpes simplex virus type 2 (HSV-2) thymidine kinase gene and predicted amino acid sequence of thymidine kinase polypeptide and its comparison with the HSV-1 thymidine kinase gene," see page 131, column 1, the abstract no. 62615w, Biochim. Biophys. Acta 1983, 741(2): 158-70 (Eng.).	86
X	Chemical Abstracts, Volume 100, No. 15, issued 1984, April 9 (Columbus, Ohio, USA), N. Sh. Alieva, "Physiological and biochemical characteristics of cotton varieties differing in their resistance to wilt," see page 327, column 1, the abstract no. 117970r, K. Izuch. Rezist. Rast. Ekstremal'nykh Vozdeistv. Sredy 1982, 44-51 (Russ).	80,84
X	Chemical Abstracts, Volume 103, No. 11, issued 1985, September 16 (Columbus, Ohio, USA), Mace et al., "Toxicity and role of terpenoid phytoalexins in verticillium wilt resistance in cotton," see page 379, column 1, the abstract no. 85240w, Physiol. Plant Pathol. 1985, 26(2): 209-218 (Eng.).	80,84

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Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Chemical Abstracts, Volume 100, No. 23, issued 1984, June 04 (Columbus, Ohio, USA), Khamraev et al., "Potassium fertilization: yield and quality," see page 499, column 2, the abstract no. 190836f, Khlopkovodstvo 1984, 3: 24-5 (Russ.).	81
X	Chemical Abstracts, Volume 105, No. 8, issued 1986, August 25 (Columbus, Ohio USA), Sarmina et al., "Effect of exposure to energy on the physicommechanical properties of cotton fibers treated with trace elements," see page 76, column 1, the abstract no. 62035k, Izv. Akad. Nauk Tadzh. SSR, Otd. Fiz.-Mat., Khim. Geol. Nauk 1985, 4: 20-5 (Russ.).	81
X	Chemical Abstracts, Volume 96, No. 15, issued 1982, April 12 (Columbus, Ohio, USA), Ryan et al., "The metabolism of chlortoluron, diuron, and CGA 43 057 in tolerant and susceptible plants," see page 236, column 1, the abstract no. 117559n, Pestic. Biochem. Physiol. 1981, 16(3): 213-21 (Eng.).	82
X	Chemical Abstracts, Volume 100, No. 11, issued 1984, March 12 (Columbus, Ohio, USA), Khuzhanazarov et al., "Effect of copper and zinc in basic fertilizer on the productivity of thin-fibered cotton," see page 468, column 1, the abstract no. 84740k, Khim. Sel'sk Khoz. 1983, 12: 21-2 (Russ.).	83

Attachment to Form PCT/ISA/210  
Part II. FIELDS SEARCHED TERMS

Cotton  
Gossypium  
regenerat?  
somatic  
transform?  
Agrobacterium  
resist or toleran?  
verticillium, improve?  
or superior  
fiber#  
or fibre#  
plant  
herbicide#  
increas?  
improv?  
yield#  
thymidine  
kinase  
gene#  
terminat?  
inventors names

Attachment to Form PCT/ISA/210Part VI. OBSERVATIONS WHERE UNITY OF INVENTION IS  
LACKING

The claims of these eight groups have the characteristics of eight distinct inventive concepts. The methods of groups I and II are distinct in that they recite different process steps and operating parameters, and produce different end products. The products of groups IV-VII are distinct from the method of group I in that plant products with the recited properties can be produced by other processes, such as by chemical or physical mutagenesis, followed by selection. The method of group I can also produce plants with a wide variety of traits other than those of the plants of these groups. The product of group III is distinct from the method of group II in that antibiotic resistant plants can be produced by methods other than transformation, such as by chemical or physical mutagenesis, and selection. The products of groups III-VII are distinct in that each possesses a unique phenotype. Finally, the product of group VIII is distinct from the method of group II in that the vectors recited can be used to transform a wide variety of plants other than cotton, and in that a wide variety of other vector constructs can be employed in the method of group II to produce transformed cotton plants.

Continuation to Attachment Form PCT/ISA/210,  
Part VI. OBSERVATION WHERE UNITY OF INVENTION IS  
LACKING:

- Group I: Claims 1-57, drawn to a method for regenerating cotton from explant material via callus; Class 435/240.5.
- Group II: Claims 58-78, drawn to a method for transforming cotton; Class 435/172.3.
- Group III: Claim 79, drawn to antibiotic resistant cotton plants; Class 800/1.
- Group IV: Claims 80 and 84, drawn to fungal resistant cotton plants; Class 800/1.
- Group V: Claim 81, drawn to cotton plants with improved fiber; Class 800/1.
- Group VI: Claim 82, drawn to cotton plants exhibiting herbicide tolerance; Class 800/1.
- Group VII: Claim 83, drawn to cotton plants exhibiting increased yield; Class 800/1.
- Group VIII: Claim 85-86, drawn to a transformation vector; Class 435/320.